LI. HAEMOGLOBIN IN RELATION TO OTHER METALLO-HAEMATOPORPHYRINS

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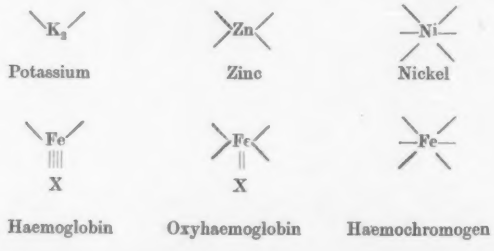
The object of this communication is to describe some observations which seem to throw some light on the union of the pigment to the protein in haemoglobin. The absorption spectra of different derivatives of haematoporphyrin are compared with those of haemoglobin, oxyhaemoglobin and haemochromogen. Then follow some experiments on the action of alkali on haemoglobin showing the formation of a pigment still containing the protein, but having the spectrum and properties of haemochromogen; on removal of the alkali the haemoglobin is regenerated. Finally some experiments on haemochromogen and on its ester are described showing their similarity in properties and the independence of the presence of water as a solvent in the formation of the compound of the ester with carbon monoxide.

Many of the metallic derivatives of haematoporphyrin have been described and the close resemblance in spectrum between the zinc compound and oxyhaemoglobin has long been known [Schultz, 1904]. The tin compound was prepared by Milroy [1909] and is noteworthy for its great stability to acids, and for the sharpness of its absorption bands which far exceeds that of the other metallic compounds. In the present communication an aluminium compound is described which closely resembles the tin compound. The fact that the cobalt compound of haematoporphyrin having a two-banded spectrum can be reduced to a one-banded form was discovered by Laidlaw [1904]; it was found that the reduced cobalt compound was similar to the nickel compound and under the conditions here described actually showed two bands. The variety of properties and spectra shown by metallic derivatives of haematoporphyrin seems to deserve a collected description and a plate of spectra has been included.

It is apparent that three groups typified by acid haematoporphyrin, oxyhaemoglobin and haemochromogen, are present. They are independent of the valency of the metal in combination, as can be seen by noting the difference between silver and sodium, and the similarity between zinc, aluminium and tin. This could be explained most simply by supposing the spectrum to be an indication of the co-ordination number of the metal, with reference to the

pigment. Starting with the above assumption an explanation of the differences in spectrum between haemochromogen, oxyhaemoglobin and haemoglobin, can be reached. Zinc and magnesium are given a co-ordination number of 4, nickel and copper 6. Hydrogen and the alkali metals are supposed to exhibit but a slight tendency to complex salt formation and form a group apart. It will be noted that the α band becomes less intense as we pass from sodium to potassium and haemoglobin itself can be regarded as the limit to this series. This would mean that in haemoglobin the iron is only attached to the pigment by its two normal valencies. When alkali acts on haemoglobin and the spectrum and properties of haemochromogen appear the iron is completely attached to the pigment and has a co-ordination number of 6. When the alkali is removed the spectrum and characteristic properties of haemoglobin are recovered; this seems to show a definite action of the globin on the iron atom, which is temporarily destroyed by the action of alkali. In oxyhaemoglobin, according to the spectrum, the iron would have a co-ordination number of 4 with reference to the pigment. If the oxygen molecule is combined with the iron atom, which has a co-ordination number of 6, no direct action of the globin on the iron atom could occur. This conclusion is supported by the fact that oxyhaemoglobin is not acted on by alkali under conditions which cause the formation of haemochromogen from haemoglobin. The fact that haemochromogen and its ester form carbon monoxide compounds leads to the supposition that there are two forms of haemochromogen, a form in which the iron is wholly combined with the pigment and which is oxidised to haematin, and a form similar to haemoglobin, which yields the carbon monoxide compound, and from which the iron is readily split off by acid. In "ordinary" haemochromogen, if there is an equilibrium between the two forms, it is easily seen that the action of oxygen must produce, in the end, nothing but haematin. In haemoglobin there can be under ordinary conditions no possibility of the oxidisible form of haemochromogen being produced. Haemochromogen, apart from globin, cannot, so far as is known, be made to show a haemoglobin spectrum; the action of globin is apparently specific. Conversion of the globin into metaprotein can readily take place without the pigment being split off; the compound (kathaemoglobin) on reduction has only the properties of haemochromogen as regards oxygen.

These assumptions can be represented graphically as follows:



1. Metallic Derivatives of Haematoporphyrin.

Preparation of haematoporphyrin. The haematoporphyrin used in the following experiments was prepared by the method of Nencki. After solution of recrystallised haemin in hydrobromic and acetic acids and hydrolysis of the addition product the pigment was precipitated from solution by adding concentrated sodium acetate solution containing about 10 % of tartrate. The pigment could then be readily filtered off and after washing with distilled water all the iron was found in the filtrate. The washed precipitate was dissolved in $N/20$ hydrochloric acid and reprecipitated with the minimum amount of Rochelle salt. This method of separating the iron was found preferable to the co-precipitation of the iron and pigment with sodium acetate, and separation from the gelatinous ferric hydroxide by solution in caustic soda.

Haematoporphyrin itself does not correspond with the iron-free pigment of haemoglobin; the relation of haematoporphyrin to the true iron-free pigment has been elucidated by H. Fischer and Müller [1925]. The difference in the position of the absorption bands between haemochromogen and the artificial compound from haematoporphyrin [Laidlaw, 1904] is very clearly shown in reference to the plate. The type of spectrum remains the same however; and when a qualitative study is being made haematoporphyrin serves for the purpose.

The introduction of metals. There are many methods of introducing a foreign metallic atom into the iron-free blood pigment, and different metals vary as to their ease of substitution. Of all the metals tried zinc was found to give the haematoporphyrin derivative most easily [Schultz, 1904]. The most general methods are: (1) to act on haematoporphyrin in glacial acetic acid with the acetate of the metal at 80°; (2) to heat the pigment to 80° for 5–10 minutes with an ammoniacal solution of a metallic salt. By method (1) manganese, iron (ferric), cobalt, nickel, copper, zinc and tin could be substituted; by method (2) iron (ferrous), cobalt, copper and lead. Magnesium can be introduced into haematoporphyrin ester by acting on it with the Grignard reagent, or the iron can be replaced directly by magnesium if haematin ester is used.

Aluminium. As the iron is tervalent in haematin and no other compound of the pigment containing a tervalent element was available, attempts were made to prepare a chromium or aluminium compound. All methods on similar lines to those adopted in the preparation of the other pigments failed and at present a chromium compound has not been produced. It was found however that by treating haematoporphyrin with pyridine saturated with anhydrous aluminium chloride at 60° a new pigment containing aluminium was produced. An examination of its properties shows that it must be regarded as the normal compound of the pigment with aluminium. It has a brilliant magenta colour and closely resembles the magnesium compound in its spectrum and shows a similar dusky yellow fluorescence. It is very soluble in both water and organic solvents, but the solution on standing gradually decomposes

with the production of brown resinous material. Like the tin compound described by Milroy [1909], its most remarkable property is its stability to acids. The metal is removed from the magnesium compound by the weakest acids, but the aluminium compound can withstand the action of concentrated sulphuric acid, and of hydrobromic in acetic acid. The solution in concentrated sulphuric acid has a blue colour which only shows a broad region of absorption in the red, but on dilution with water the original colour and absorption spectrum return. The tin compound, like acid haematoporphyrin, dissolves in concentrated sulphuric acid without change of spectrum. When arsenic trichloride is substituted for aluminium chloride under the above conditions a solution of a deep yellowish-brown colour is produced. The spectrum resembles that of alkaline haematin; on pouring into alcohol a solution of acid haematoporphyrin is at once obtained showing that no permanent alteration in the pigment has taken place.

Alkali metals. Acid haematoporphyrin itself must be regarded as the hydrogen compound corresponding to the other metallic derivatives showing the characteristic two-banded spectrum. This is justified by an examination of the sodium and potassium compounds. The alkali metals can be introduced into haematoporphyrin by shaking a solution of the pigment in dry pyridine containing 5 % of methyl alcohol with an excess of powdered sodium or potassium hydroxide; the red-brown colour of the haematoporphyrin changes to a bright magenta in the case of the sodium and to red-purple in the case of the potassium. Traces of water or even alcohol added to the solution at once remove the alkali metal yielding ordinary alkaline haematoporphyrin.

By shaking a solution of alkaline haematoporphyrin with silver oxide or carbonate preferably in the presence of alcohol, silver is introduced and the solution shows a spectrum similar to that of the copper compound. Thus the univalent silver atom when introduced shows no similarity with the alkali metals. The stability of the silver compound to light and most reagents is of interest. Hydrochloric acid only slowly liberates haematoporphyrin, silver chloride being precipitated.

Cobalt. Of all the metallic derivatives of haematoporphyrin examined only those with iron and cobalt show a reduction and oxidation effect. Laidlaw [1904] found that the ordinary two-banded cobalt derivative was reduced to a one-banded form. By acting on a solution of the cobalt derivative in caustic soda with hydrazine hydrate it was reduced to a form showing two bands, one much stronger than the other and similar to those of the nickel compound. Thus this reduced form of the cobalt haematoporphyrin is probably the normal cobaltous compound corresponding to that of nickel. Oxidation to the original two-banded form took place with great ease, and the reduction could only be made to occur in strongly alkaline solution. By acting on the reduced form with nitric oxide a spectrum similar to that of the oxidised form was produced which did not change when treated with the reducing agent. As Laidlaw showed, there was no reaction with carbon monoxide. The oxidised form of the

cobalt derivative shows little resemblance to oxyhaemoglobin. The substance is unaffected by hydrochloric acid and organic solvents, and the oxygen is not removed by a vacuum and only by the action of a powerful reducing agent. It seems clear that it involves a change from the cobaltous to cobaltic state and possibly more nearly resembles the oxidation of haemochromogen to haematin.

Collective properties of these compounds. When the absorption spectra of a series of metallic derivatives of haematoporphyrin are examined, those whose solutions have a red or purple colour can be placed in three groups which do not depend on the valency of the metal. For the type of each group we can take (1) acid haematoporphyrin, (2) oxyhaemoglobin and (3) haemochromogen. In group (1) the α band is less developed than the β band and the colour is usually purplish red or purple. In group (3) the α band is more strongly developed than the β band and the colour is usually scarlet red. Group (2) is intermediate in character. The main properties of metallic derivatives of haematoporphyrin are summarised in the following table. The figures refer to the above types of spectrum, whilst the letter H indicates a type of haematin spectrum. The property of dissolving in acetate buffer shows that the normal solubilities of the pigment are strongly influenced by combination with the metal. The ease with which the metal is removed by different acids is indicated by a positive or a negative sign.

			Metal removed by			
Metal	Type of spectrum	Soluble in acetate buffer	Water	Dilute acetic acid	Hydro-chloric acid	Conc. sulphuric acid
Na	1	.	+	.	.	.
K	1	.	+	.	.	.
Ag	3	−	−	−	+	.
Mg	2	−	−	+	.	.
Zn	2	−	−	−	+	.
Al	2	+	−	−	−	−
Mn	H	+	−	−	−	+
$Fe^{\cdot\cdot}$	3	−	−	−	±	.
$Fe^{\cdot\cdot\cdot}$	H	−	−	−	−	+
$Co^{\cdot\cdot}$	3	.	−	.	.	.
$Co^{\cdot\cdot\cdot}$	2	+	−	−	−	+
Ni	3	−	−	−	−	+
Cu	3	−	−	−	−	+
As	H	.	+	.	.	.
Sn	2	+	−	−	−	−
Pb	H	.	−	+	.	.

2. The action of Strong Alkali on Haemoglobin.

In some unpublished observations J. S. Haldane found that when haemochromogen was prepared by adding caustic soda to reduced blood the mixture would, on standing, revert to haemoglobin. Experiments were tried in imitation of this, using different concentrations of alkali, and ferrous sodium tartrate as a reducing agent. In $N/2$ alkali the reduced haemoglobin shows a strong haemochromogen spectrum but the solution on dilution with water

(being kept reduced) departs widely from Beer's absorption law, equivalent thicknesses, showing less of the haemochromogen spectrum as dilution proceeds until finally the absorption due to reduced haemoglobin becomes dominant. This clearly shows the reversible nature of the action of alkali.

When the solution of reduced haemoglobin in $N/2$ alkali is oxidised haematin results together with oxyhaemoglobin. It was found that, though the solution showed the haemochromogen bands only, about 50 % of the haemoglobin remained undecomposed. If, however, the solution in $N/2$ alkali was warmed it became of a lighter scarlet colour, and showed no alteration of absorption spectrum on dilution. When oxidised it yielded haematin alone—whether diluted or undiluted—showing a complete and irreversible decomposition into haemochromogen. Experiments were then tried using stronger solutions of alkali and all the solutions were cooled. As the experiments are only concerned with the *reversible* action of alkali, precautions had to be taken to avoid irreversible haemochromogen formation taking place. It was not found possible under the conditions used completely to eliminate this action, but it could be reduced to a low enough level not to interfere with qualitative experiments. It was found that minute traces of almost any foreign organic material—cork, wax, filter paper, or organic solvents in the soda solution—would cause an almost instantaneous irreversible haemochromogen formation, especially when the concentration of alkali added was high. In the absence of such impurities no such rapid action took place. Similarly hydrosulphite could not be used as a reducing agent; fortunately alkaline ferrous tartrate did not prove injurious even in a large excess.

The experiments were of the following type—a 1 % solution of haemoglobin either from washed corpuscles or defibrinated blood was reduced and cooled; to it was added a cooled solution of 40 % sodium hydroxide containing a trace of the reducing agent (ferrous tartrate) until a definite concentration of sodium hydroxide was reached; the mixture was then examined. At – 5° conversion into haemochromogen was not complete until the solution contained 20 % of sodium hydroxide. Such a solution had, when reversible, a scarlet colour, more brilliant than when the solution was irreversible; no difference has been detected up to the present in the bands, and on oxidation both are completely converted into alkaline haematin. The solution in both cases seems to contain ordinary haemochromogen. The change from reversibility to irreversibility must be due to a change in the protein—as is evidenced by a turbidity which often shows itself. To test the mixtures for reversibility they were diluted with an ice-cold solution of an ammonium salt (either sulphate or chloride) to which a little Stoke's reagent had been added. The soda is thereby replaced by ammonia which has comparatively little action on haemoglobin. Under these conditions the scarlet colour of the haemochromogen changed to the purple of haemoglobin, and the change was usually almost complete in 5 minutes. If irreversible haemochromogen formation had taken place the solution, instead of being clear, was turbid and remained

scarlet indefinitely. The oxyhaemoglobin obtained after the alkali treatment had an absorption spectrum identical with that of the untreated substance, no shift in the bands being observable.

When the amount of alkali was increased so that there was 30 % in solution the pigment was completely precipitated as a scarlet flocculent mass, still showing the haemochromogen spectrum. The mixture could be converted into a clear solution of haemoglobin on treatment with an ammonium salt. If, however, it was allowed to warm to room temperature the precipitate swelled up slightly and became of a paler colour; it was then insoluble in the solution of an ammonium salt and gave no haemoglobin. When a solution of haemoglobin from twice washed corpuscles was used for the experiment the precipitate had a decidedly crystalline appearance and was intensely scarlet. A small quantity was filtered on asbestos in a Gooch crucible surrounded by an ice and salt jacket and washed twice with 40 % sodium hydroxide. The washings were colourless. On treating the residue with dilute ice-cold ammonium sulphate solution it partially dissolved forming a clear solution of haemoglobin. The insoluble portion consisted of coagulated protein and haemochromogen. From the homogeneous appearance of the precipitate obtained by the action of 30 % sodium hydroxide and from the fact that it will yield haemoglobin after washing with 40 % sodium hydroxide, it is concluded that it is a compound of haemochromogen and globin, probably of a colloidal nature. In solution in 20 % sodium hydroxide the haemochromogen cannot be precipitated in the presence of a calcium salt, whilst in the absence of globin no haemochromogen can remain in solution.

Thus, whilst the *specific* action of globin on haemochromogen is temporarily inhibited by alkali, the protein itself is capable of resisting the reagent. It now became important to examine the behaviour of oxyhaemoglobin and carboxyhaemoglobin under the same conditions. It is usually believed that oxyhaemoglobin yields haematin with moderate concentrations of alkali, but under the above conditions this is found not to be the case. Oxyhaemoglobin remained unaltered in colour and absorption spectrum in solutions of sodium hydroxide of concentrations up to 30 % and precipitation of the pigment did not occur. Carboxyhaemoglobin behaved similarly. When the solution of oxyhaemoglobin containing alkali was submitted to a vacuum both haematin and haemochromogen were formed, showing that the oxygen was still dissociable and that the haemoglobin, presumably formed, suffered decomposition by the alkali into haemochromogen which then became partially oxidised.

3. Haemochromogen in Pyridine.

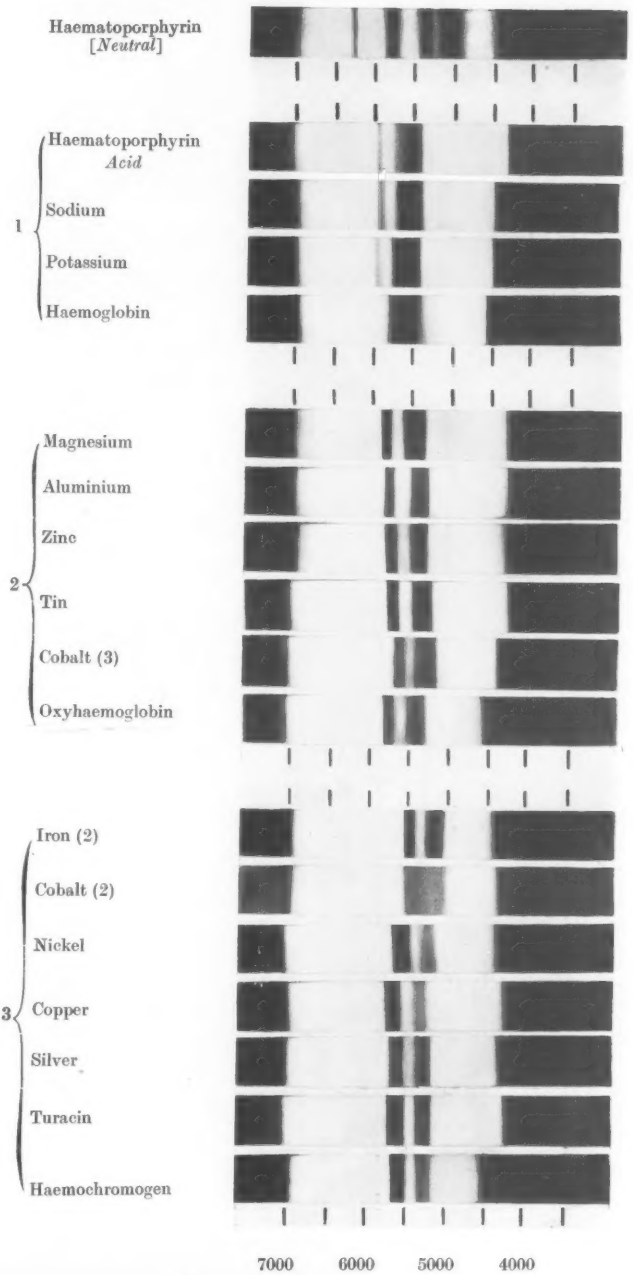
A solution of haemochromogen in dry pyridine can readily be prepared. Whereas haemin dissolves in the moist solvent with a red-brown colour (giving the characteristic haematin-pyridine spectrum), in the dry solvent a brown solution is obtained having the absorption spectrum of acid haematin. This

brown solution is completely reduced to haemochromogen on shaking for 1 minute with metallic mercury. Haemin thus resembles ferric chloride in the ease with which a chlorine atom is removed when dissolved in a dry organic solvent, the iron being reduced to the ferrous state. If haemin is added to dry piperidine, haemochromogen is at once produced without the addition of mercury.

The solution of haemochromogen in dry pyridine was bright scarlet and stable in air, not forming haematin as in the case of the aqueous solutions. Dry oxygen was passed through this solution at room temperature for 1 hour, and no change was observed either in the colour of the solution or in its absorption spectrum. The resistance to oxidation was diminished as increasing amounts of water were added. If an equal volume of distilled water was added the haemochromogen was completely converted into haematin. Coal gas passed through the solution produced no change in the absorption spectrum. Thus both the oxidation of haemochromogen to haematin and the formation of the carbon monoxide compound are prevented by pyridine, and if there is an oxygen compound analogous to that with carbon monoxide it might be thought to play a part in the oxidation of haemochromogen to haematin.

4. Methyl ester of Haemochromogen.

Amorphous haematin ester was obtained by washing an ethereal solution of haematin, methylated with dimethyl sulphate, with sodium carbonate solution; after drying with anhydrous sodium sulphate the solid ester was obtained by distilling off the ether. The dried product was dissolved in absolute alcohol and treated with the minimum quantity of hydrazine hydrate in an atmosphere of coal gas. Immediate reduction and rapid conversion of the haemochromogen ester into the carbon monoxide compound took place. The solution was warmed to 60° and diluted with water until precipitation of the pigment commenced. After cooling the solid was filtered off in an atmosphere of coal gas and washed with distilled water saturated with coal gas and containing a trace of hydrazine hydrate. It was dried in coal gas, all contact with air being avoided until the moisture had all been removed. The compound consisted of a pure Bordeaux-red powder, made up of minute hexagonal plates. It is soluble in all the usual organic solvents forming brilliant carmine red solutions which decompose in a few seconds, forming dull-brown solutions of haematin ester. If the solvent is carefully dried the action is retarded, but oxygen and moisture from the air rapidly produce oxidation. The absorption spectrum agrees closely with that of carbon monoxide haemoglobin. When the carbon monoxide ester of haemochromogen was dried over P_2O_5 in coal gas and dissolved in dry xylene, a stable carmine solution was obtained if air was excluded. But on passing dry oxygen the solution at once turned brown and was found to contain haematin ester. Even when great precautions were taken to avoid the presence of moisture haematin was produced by the action


Haematoporphyrin [Neutral]
1
Haematoporphyrin Acid
Sodium
Potassium
Haemoglobin
2
Magnesium
Aluminium
Zinc
Tin
Cobalt (3)
Oxyhaemoglobin
3
Iron (2)
Cobalt (2)
Nickel
Copper
Silver
Turacin
Haemochromogen
7000
6000
5000
4000

of oxygen. The solid ester compound could not be kept; even over P_2O_5 it was gradually converted into a black powder.

It was found that pyridine not only prevents the formation of the carbon monoxide compound of haemochromogen but also causes the decomposition of that substance. When a little of the dry carbon monoxide compound was added to dry pyridine, the substance dissolved with vigorous effervescence forming a scarlet solution having the absorption spectrum of haemochromogen.

A concentrated solution of oxyhaemoglobin was prepared by centrifuging blood and laking the corpuscles with ether. One drop of this was added to 2 cc. of dry pyridine in a test-tube. Protein was precipitated and in the precipitate bubbles of gas were entangled. On standing 1 minute the clear liquid was scarlet red and showed the absorption spectrum of haemochromogen. Methaemoglobin prepared with ferricyanide and dialysed did not produce a trace of haemochromogen when added to pyridine but simply the brown-red solution of haematin.

Summary.

1. Metallic derivatives of haematoporphyrin showing two-banded spectra can be divided into three classes which are independent of the valency of the metal.

2. Haemoglobin is completely changed to a substance having the spectrum and properties of haemochromogen by high concentrations of alkali. In the cold the action is reversible.

3. Oxyhaemoglobin and carbon monoxide haemoglobin are unaltered by high concentrations of alkali in the cold.

4. Haemochromogen in dry pyridine is not acted on by free oxygen, neither is a carbon monoxide compound formed.

5. Haemochromogen ester in organic solvents shows the characteristic reactions of the free substance.

6. Pyridine causes the decomposition of the carbon monoxide compound into free haemochromogen ester. Pyridine forms haemochromogen from oxyhaemoglobin as well as from haemoglobin.

I wish to thank Sir F. G. Hopkins for his kind encouragement and advice, and Dr Haldane for his private communication. I desire also to express my gratitude to the Department of Scientific and Industrial Research for grants held during the period of this research.

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LII. THE SPECIFIC ACTION OF PLANT FERMENTS.

I. THE SPECIFIC CONDITIONS OF ACTION OF LEAF INVERTASES.

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The investigations of Sörensen [1909], Michaelis and Davidsohn [1911] and Euler and Emberg [1919] on the optimal p_H-values for the action of yeast-invertases, and those of Euler and Svanberg [1921] for the action of intestinal invertase of man, gave a distinct dissimilarity of these values: the greatest action of yeast-invertase was found at p_H 3·5–5·5; that of intestinal invertase at p_H 5–7. That the invertases from various yeasts are not identical is made clear by Willstätter and Kuhn [1923].

Our experiments on the invertases of leaves of various plants give a new confirmation for the Willstätter point of view, according to which we have as many invertases as sources from which they are obtained.

We investigated the water extracts from air-dried powdered leaves of 14 species of wild and cultivated plants. The leaves were taken under identical conditions from various individuals of the same species (July–August, 1924), dried rapidly at 25–30° and ground in a mortar. 1 g. of a leaf-powder was mixed with 60 cc. of water and filtered through a dry filter after standing at 25° during 24 hours with toluene. 5 cc. of clear filtrate were mixed with 10 cc. of phosphate buffer mixture and 5 cc. 8 % saccharose solution. To all experimental flasks toluene was added and they were kept at 25°. The determinations of invert-sugar were made by the Benedict-Lewis picric acid method. The standard solutions were prepared each week according to Willaman and Davison's [1924] directions, with intervals of 0·125 mg. of invert-sugar. For each determination 0·5 cc. of the solution to be investigated was taken. The duration of each single experimental series varied from 23 to 91 hours at about 30°.

Exp. 1. The leaves of *Prangos pabularia* Lindl., a species of the Umbelliferae indigenous to the valleys of West Tian-Shan, were investigated at the Botanical Station of the University of Chimgan. The initial content of reducing sugar in 0·5 cc. of the experimental solution is 1·0 mg.; duration of hydrolysis 47½ hours.

p_H	Reducing sugar mg. in 0·5 cc.	Change mg.	Change %
4·5	3·50	+2·50	48
5·0	4·75	3·75	71
5·6	5·25	4·25	81
5·9	5·50	4·50	86
6·2	5·75	4·75	90
6·5	6·00	5·00	95
6·6	6·25	5·25	100
7·0	5·25	4·25	81

Exp. 2. The subterranean leaves of bulbs of *Ungernia Severzovii* (Rgl.) B. Fedtsch., a species of the Amaryllidaceae from West Tian-Shan. The initial content of reducing sugar in 0·5 cc. of the experimental solution is 1·125 mg.; duration of hydrolysis 49½ hours.

p_H	Reducing sugar mg.	Change mg.	Change %
4·5	1·425	0·300	91
5·0	1·450	0·325	100
5·6	1·425	0·300	91
5·9	1·300	0·125	39
6·2	1·250	0·100	33
6·5	1·150	0·025	9
6·6	1·125	0·000	0

Exp. 3. The leaves of *Juglans fallax* Dode, from the Chimgan valley in West Tian-Shan. The initial content of reducing sugar is 0·650 mg.; duration of hydrolysis 46½ hours.

p_H	Reducing sugar mg.	Change mg.	Change %
4·5	3·750	3·100	55
5·0	4·250	3·600	64
5·6	5·250	4·600	82
5·9	6·250	5·600	100
6·2	5·750	5·100	91
6·5	5·000	4·350	78
6·6	3·750	3·100	55
7·0	2·750	2·100	38

Exp. 4. The leaves of *Vitis vinifera* L. cultivated at Tashkent. The initial content of reducing sugar in 0·5 cc. of the experimental solution is 0·525 mg.; duration of hydrolysis 48 hours.

p_H	Reducing sugar mg.	Change mg.	Change %
4·4	1·150	0·625	64
4·5	1·450	0·925	95
5·0	1·500	0·975	100
5·6	1·375	0·850	87
5·9	1·375	0·850	87
6·2	1·225	0·700	72
6·5	1·100	0·575	59
6·6	0·475	0·475	49
7·0	0·525	0·000	0

Exp. 5. The leaves of *Gossypium herbaceum* L. from the Turkestan Plant Breeding Station at Tashkent. The initial content of reducing sugar is 0·375 mg.; duration of hydrolysis 41 hours.

	Reducing sugar	Change	
p_H	mg.	mg.	%
4·0	0·650	0·275	31
4·4	0·800	0·425	49
4·5	1·150	0·775	89
5·0	1·150	0·775	89
5·6	1·250	0·875	100
5·9	1·175	0·800	91
6·2	1·050	0·675	77
6·5	0·975	0·600	69
6·6	0·750	0·375	43
7·0	0·800	0·425	49

Exp. 6. The leaves of *Gossypium hirsutum* L. from the Turkestan Breeding Station at Tashkent (strains No. 182 and No. 509). The initial content of reducing sugar for No. 182 is 0·875 mg. and for No. 509 1·100 mg.; duration of hydrolysis for No. 182 46½ hours and for No. 509 45 hours 50 minutes. For p_H 4·0 and 4·4 glycine buffer solutions were used.

	No. 182			No. 509		
	Reducing sugar	Change		Reducing sugar	Change	
p_H	mg.	mg.	%	mg.	mg.	%
4·0	0·875	0·0	0	1·100	0·0	0
4·4	0·875	0·0	0	1·100	0·0	0
4·5	1·150	0·275	79	1·600	0·500	91
5·0	1·225	0·350	100	1·650	0·550	100
5·6	1·200	0·325	93	1·500	0·400	73
5·9	1·175	0·300	86	1·450	0·350	64
6·2	1·125	0·250	71	1·375	0·275	50
6·5	1·100	0·225	64	1·250	0·150	27
6·6	1·000	0·125	36	1·175	0·075	18
7·0	0·950	0·075	22	1·225	0·125	23

Exp. 7. The leaves of *Glycyrrhiza glabra* L. from Chimgan. The initial content of reducing sugar is 0·500 mg.; duration of hydrolysis 91 hours.

	Reducing sugars	Change	
p_H	mg.	mg.	%
4·0	0·500	0·0	0
4·4	0·500	0·0	0
4·5	0·900	0·400	100
5·0	0·850	0·350	88
5·6	0·775	0·275	69
5·9	0·600	0·100	25
6·2	0·525	0·025	6
6·5	0·500	0·0	0

Exp. 8. The leaves of *Platanus orientalis* L. from the University Botanical Garden at Tashkent. The initial content of reducing sugar is 0·625 mg.; duration of hydrolysis 47 hours 45 minutes. For p_H values from 3·3–4·0 glycine buffer solutions were used.

	Reducing sugar	Change	
p_H	mg.	mg.	%
3·3	0·925	0·300	34
3·7	1·250	0·625	72
4·0	1·250	0·625	72
4·5	1·500	0·875	100
5·0	1·425	0·800	92
5·6	1·375	0·750	86
6·2	1·250	0·625	72
6·6	0·900	0·275	32
7·0	0·875	0·250	29

Exp. 9. The leaves of *Pyrus communis* L. (Tashkent). The initial content of reducing sugar is 0·550 mg.; duration of hydrolysis 39 hours.

	Reducing sugar	Change	
p_H	mg.	mg.	%
4·5	2·250	1·700	60
5·0	2·375	1·825	65
5·6	2·750	2·200	78
5·9	3·200	2·650	94
6·2	3·375	2·825	100
6·5	3·000	2·450	87
6·6	1·825	1·275	45
7·0	1·425	0·875	31

Exp. 10. The leaves of *Pyrus malus* L. (Tashkent). The initial content of reducing sugar is 0·825 mg.; duration of hydrolysis 49½ hours.

	Reducing sugar	Change	
p_H	mg.	mg.	%
4·4	4·750	3·925	72
4·5	5·875	5·050	93
5·0	6·160	5·325	98
5·6	6·250	5·425	100
5·9	5·950	5·125	95
6·2	5·500	4·675	86
6·5	4·625	3·800	70
6·6	3·500	2·675	49
7·0	2·625	1·800	33

Exp. 11. The leaves of *Quercus pedunculata* L. (Tashkent). The initial content of reducing sugar is 0·775 mg.; duration of hydrolysis 23 hours.

	Reducing sugar	Change	
p_H	mg.	mg.	%
4·5	5·250	4·475	88
5·0	5·375	4·600	90
5·6	5·500	4·725	93
5·9	5·625	4·850	95
6·2	5·750	4·975	98
6·5	5·875	5·100	100
6·6	5·375	4·600	90
7·0	2·625	1·850	58

Exp. 12. The leaves of *Syringa vulgaris* L. (Tashkent). The initial content of reducing sugar is 0·675 mg.; duration of hydrolysis 38½ hours.

	Reducing sugar	Change	
p_H	mg.	mg.	%
4·5	1·075	0·400	89
5·0	1·025	0·350	76
5·6	1·025	0·350	76
5·9	1·050	0·375	83
6·2	1·125	0·450	100
6·5	1·050	0·375	83
6·6	1·075	0·400	89
7·0	0·950	0·275	61

Exp. 13. The leaves of *Fraxinus* sp. (Tashkent). The initial content of reducing sugar is 0·350 mg.; duration of hydrolysis 62 hours.

	Reducing sugar	Change	
p_H	mg.	mg.	%
4·5	1·450	1·100	92
5·0	1·475	1·125	94
5·6	1·500	1·150	96
5·9	1·500	1·150	96
6·2	1·550	1·200	100
6·5	1·500	1·150	96
6·6	1·300	0·950	79
7·0	1·200	0·850	71

Exp. 14. Finally, we investigated the expressed juice of *Salicornia herbacea* L. (from the salt desert Golodnaja Steppe). No reducing sugar at start; duration of hydrolysis 64½ hours.

	Reducing sugar	Change	
p_H	mg.	mg.	%
4·5	0·950	0·950	68
5·9	1·200	1·200	86
6·2	1·400	1·400	100
6·5	1·250	1·250	90
6·6	1·175	1·175	84
6·8	1·125	1·125	80
7·0	1·025	1·025	73
7·2	0·975	0·975	70
7·4	0·875	0·875	63
7·7	0·925	0·925	66
7·9	0·800	0·800	57
8·0	0·625	0·625	45

All our experiments give a definite conclusion that a definite optimal hydrogen ion concentration exists for each plant species and that variation of ratio of hydrolysis with change in p_H is of a very specific character.

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LIII. THE SPECIFIC ACTION OF PLANT FERMENTS.

II. THE SPECIFIC CONDITIONS OF ACTION OF LEAF PEPTASES.

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The investigations of Blagoveschenski and Sossiedov [1925] on the specific conditions of action of leaf invertases of various plant species have shown that each of these has a specific optimal p_H-value for its action. In the present paper we communicate our experiments on the action of plant peptases at various hydrogen ion concentrations.

We took samples of dried and finely ground leaves (exactly 1 g.) and mixed these with 10 cc. of 8 % peptone solution, 10 cc. of buffer (phosphate or glycine) mixture and some drops of toluene. All the flasks (two for each p_H-value) were kept in the thermostat at 25° or 30° for 72 hours. Each experimental series was followed by a control series without ferment action. The hydrolysis of peptone was measured by an amino nitrogen determination by Sörensen's method. The phosphates were removed with barium chloride and barium hydroxide. The formaldehyde titration was carried out with a comparator, for the elimination of the colour of the leaf extracts. The leaves were collected partly at Chimgan Botanical Station (*Astragalus Sieversianus* Pall., *Ungernia Severzovii* (Rgl.) B. Fedtsch., *Juglans fallax* Dode, *Prangos pabularia* Lindl., *Glycyrrhiza glabra* L.), partly in Tashkent Botanical Garden (*Syringa vulgaris* L., *Vitis vinifera* L., *Pyrus malus* L., *Pyrus communis* L., *Fraxinus* sp., *Quercus pedunculata* L., *Platanus orientalis* L.). The leaves of *Gossypium herbaceum* L. and *Gossypium hirsutum* L. were collected at the fields of the Turkestan Plant Breeding Station at Tashkent.

The data given in Table I demonstrate that the optimal concentrations of hydrogen ions for the action of peptases from leaves of various plants are very varied and specific for single plant species. It is interesting that at p_H 7·1, the neutral point, we could not find an optimum for any peptase, although they occur at both higher and lower p_H values.

We made a special comparative quantitative determination of the peptase action of 11 species at their optimal p_H values (Table II).

The activity of peptase (or its amount) in various plants is also specific.

Table I.

	p_H-values	4·5	5·4	5·8	6·3	6·7	7·1	7·7	8·0
		Change of amino nitrogen content.							
1. Ungernia Severzovii	mg.	—	+0·7	2·1	3·5	3·8	3·8	5·3	6·3
	%	—	11	33	56	60	60	83	**100**
2. Astragalus Sieversianus	mg.	—	4·9	1·8	4·9	6·7	9·1	11·9	8·4
	%	—	41	15	41	56	76	**100**	71
3. Syringa vulgaris	mg.	8·4	4·2	5·6	8·4	7·0	14·0	15·4	14·0
	%	55	27	36	55	45	91	**100**	91
4. Syringa vulgaris (middle)	mg.	5·6	4·2	4·2	1·4	4·2	7·7	9·8	8·4
	%	55	43	43	14	43	78	**100**	87
	%	55	35	39	35	44	85	**100**	89
5. Vitis vinifera	mg.	5·6	3·5	4·2	7·0	9·8	9·8	11·2	9·8
	%	50	32	37	63	88	88	**100**	88
6. Juglans fallax	mg.	9·8	10·2	7·0	9·8	11·2	8·4	11·2	7·8
	%	88	91	63	88	**100**	75	**100**	88
7. Prangos pabularia	mg.	—	4·9	5·9	—	9·8	8·4	9·1	7·4
	%	—	50	60	—	**100**	82	92	76
8. Glycyrrhiza glabra	mg.	8·6	—	6·7	10·7	7·9	9·3	7·9	4·4
	%	80	—	63	**100**	74	87	74	41
9. Pyrus communis	mg.	7·7	7·7	8·4	7·0	5·6	5·6	2·8	2·8
	%	92	92	**100**	89	67	67	34	34
10. Fraxinus sp.	mg.	5·6	5·6	7·0	4·2	2·8	4·2	1·4	1·4
	%	80	80	**100**	65	40	65	20	20
11. Gossypium herbaceum	mg.	11·2	11·2	14·0	11·9	11·6	9·1	9·6	5·6
	%	80	80	**100**	85	83	65	69	40
12. Gossypium hirsutum, No. 182	mg.	10·4	11·6	14·0	8·4	7·7	7·8	7·0	5·6
	%	74	83	**100**	60	55	56	50	40
13. Gossypium hirsutum, No. 509	mg.	9·8	11·8	13·3	10·5	8·4	6·3	7·0	4·2
	%	74	84	**100**	78	70	47	53	32
14. Quercus pedunculata	mg.	23·8	—	14·0	11·2	8·4	9·8	9·8	11·2
	%	**100**	—	59	47	35	41	41	47
15. Platanus orientalis	mg.	91	7·7	5·6	1·4	1·4	1·4	0·0	0·0
	%	**100**	85	62	15	15	15	0	0
16. Pyrus malus	mg.	7·0	—	5·6	—	2·8	1·4	0	0
	%	**100**	—	80	—	40	20	0	0

Table II.

Name	p_H (optimal)	Amino N change in mg.
Pyrus malus	4·5	+9·1
Pyrus communis	5·8	12·6
Vitis vinifera	7·7	14·0
Juglans fallax	6·7	12·8
Gossypium herbaceum ...	5·8	14·7
Gossypium hirsutum, No. 182	5·8	12·6
Gossypium hirsutum, No. 509	5·8	12·0
Syringa vulgaris	7·7	7·0
Fraxinus sp.	5·8	14·0
Platanus orientalis	4·5	7·0
Quercus pedunculata ...	4·5	14·0

All these results give a new confirmation to the point of view that physiologically plants can have just as definite properties as morphologically, and we have the right to suppose from these and many other similar observations and experiments the existence of a connection between the variability of the properties of living matter and that of external form.

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LIV. THE PHYSIOLOGY OF GASTRIC UREASE.

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We have recently shown that very active preparations of urease may be obtained from the stomachs of carnivora [Luck, 1924] and that the enzyme by its behaviour *in vitro* appears to be identical with soy bean urease [Luck and Seth, 1924].

As it was possible, in the absence of evidence to the contrary, that the enzyme might be a product of *post mortem* changes it was considered advisable to demonstrate if possible its presence *in vivo*. In proceeding to do so we have assumed that the observable effects would be either a catalysis of the hydrolysis of urea or the synthesis of that substance from ammonium carbonate. By this assumption we have not overlooked a view which theoretically and teleologically is more desirable. This is that both the synthetic and hydrolysing functions may be exercised by the enzyme, the direction of the reaction being controlled by the many physico-chemical factors which influence the equilibria within the cell.

Experimentally we have investigated the effect of altering the concentrations of urea and ammonium carbonate. In the one case the concentration of urea in the cells of the mucosa was raised by the gastric absorption of urea, or, as in one experiment, by the intravenous injection of that substance. The resulting effect on the cellular concentration of ammonia was observed by estimating at suitable intervals the ammonia content of the gastric venous blood. In the second case ammonium salts in aqueous solution were introduced into the stomach and urea estimations were made upon the blood flowing from that organ. In two experiments a third factor, the hydrogen ion concentration of the cell, was varied. This was done on the strength of that once popular idea that the formation of urea would be favoured by alkalosis and that of ammonia by acidosis.

The results suggest that the enzyme functions *in vivo* in the same way that it does *in vitro*. An increased concentration of urea in the cells of the gastric mucosa was invariably accompanied by an increase in the ammonia content of the gastric venous blood. On the other hand, we were unable to observe any indication of the gastric formation of urea under the conditions of our experiments.

Experimental.

All but the preliminary observations were made upon dogs which had fasted for 48 hours before use, to ensure freedom of the stomach from food. The animal was anaesthetised with ether and a tracheal cannula was inserted. The right femoral artery was exposed and a mid-ventral incision was made through the abdominal wall. The stomach was closed distally by a pyloric ligature. The mesenteric vessels were tied and the splenic vessels were ligatured close to the spleen. By this procedure the contents of the vena lienalis were limited to the blood which entered from the left gastro-epiploic vein, and the blood of the portal vein consisted principally of that from the stomach.

The blood samples of 6 cc. were drawn by syringe and were received in tubes containing 0·6 cc. of ammonia-free 2 % potassium oxalate. The syringe was washed with freshly distilled ammonia-free water before the drawing of a sample. The arterial punctures were made distal to the profunda artery. The gastric venous blood was drawn from the portal vein close to the porta hepatis. In general, six samples in all were collected, three portal and three arterial—the first pair serving as controls. After drawing the first pair of samples the experimental fluid was administered at 38°. The second and third pairs were drawn at approximately $1\frac{1}{2}$ and $2\frac{1}{2}$ hours after administration of the fluid. In some of the experiments modifications of this procedure were necessary. Where this has been so the experimental details have been given elsewhere in the text.

As we assumed the presence of a very labile ammonia-forming substance in blood [Medwedew, 1911; Henriques and Gottlieb, 1924; Parnas and Heller, 1924] the following method of ammonia estimation was designed to reduce autolytic changes to a minimum and to permit the simultaneous collection of several samples of blood. The principle of the method was the removal of the blood proteins with tungstic acid and the estimation of the ammonia in the centrifugate by distillation.

To 5 cc. of the oxalated blood were added immediately after collection 5 cc. each of ammonia-free water, 10 % sodium tungstate and 0·066 *N* sulphuric acid. The mixture was centrifuged. The centrifugates, which measured 11–13 cc. and were water-clear, were decanted into tubes and kept at − 2 to 0° until removed for estimation.

The method of distillation was designed to minimise secondary ammonia formation by hydrolysis and to permit direct nesslerisation of low volume distillates. Stanford's apparatus [Stanford, 1923] was used. By immersing both the receiver and the trap in crushed ice and evacuating with a Geryk pump, rapid distillation could be obtained with a bath temperature of 25–30°. 5 cc. of 0·05 *N* sulphuric acid were placed in the receiver and a similar volume in the trap; 10 cc. of the centrifugate were placed in the distillation flask. When the apparatus was sufficiently evacuated 5 cc. of alkali (consisting of

4 cc. of saturated sodium carbonate and 1 cc. of saturated sodium bicarbonate) were added. Distillation was continued for 30–40 minutes, in which time 8–9 cc. of distillate were collected. A second fraction obtained by continued distillation into a fresh receiver gave no colour with Nessler's reagent. Recovery of the ammonia was therefore complete. The contents of the receiver were weighed, nesslerised with one-tenth their volume of the reagent and compared with ammonium sulphate standards.

In the estimation of blood urea the tungstic acid precipitation of Folin and Wu [1919] was employed for the removal of proteins. To 4 cc. of the filtrate were added 0·3 cc. of a molar phosphate solution of p_H 7·45 and 1 cc. of 0·5 % urease [Van Slyke and Cullen, 1914] filtered through glass wool. After standing for 30 minutes at 40° the ammonia was distilled into standard acid and estimated by nesslerisation.

All tubes, pipettes, and other pieces of apparatus were repeatedly washed with freshly prepared ammonia-free water which was obtained by the distillation of dilute sulphuric acid and was used for the preparation of all reagents. The potassium oxalate was thrice recrystallised from ammonia-free water. The sodium carbonate solution was well boiled and when cold was added to the saturated solution of sodium bicarbonate. The sulphuric acid was obtained from freshly opened supplies and was found to contain only a very small quantity of ammonia. The final quality of the reagents was controlled by blank estimations.

The ammonia content of whole blood in vitro *at* 0°.

The necessity of immediate treatment of the blood samples is shown by the rapid increase in the ammonia content of rabbit blood even at 0°. A rabbit under ether anaesthesia was bled by cannula from the abdominal aorta. 35 cc. of blood were run into 5 cc. of the oxalate solution. 5 cc. of the oxalated blood were precipitated immediately. The remainder was stored at – 2 to 0° and precipitated in 5 cc. portions at varying intervals. The ammonia content of each centrifugate was estimated immediately.

Time after collection of blood	15 min.	1 h. 5 min.	2 h.	4 h. 5 min.	7 h.	24 h.
Mg. ammonia N per 100 cc. of blood	0·22	0·31	0·43	0·57	0·68	0·81

The ammonia content of the tungstic acid centrifugates at 0°.

By immediate precipitation of the blood samples it was found that the centrifugates could be stored at 0° without any increase in the ammonia content. It was concluded that either the labile ammonia precursor was precipitated by the tungstic acid or the acidity of the centrifugate inhibited ammonia formation. 30 cc. of blood were drawn from the abdominal aorta of a rabbit and run into 5 cc. of oxalate. Six portions of 5 cc. each were immediately precipitated and centrifuged. The centrifugates were stored at – 2 to 0°.

Time after precipitation	25 min.	1 h. 40 min.	2 h. 35 min.	5 h. 40 min.	23 h. 30 min.
Mg. ammonia N per 100 cc. blood	0·11	0·08	0·09	0·11	0·10

The recovery of added ammonia.

40 cc. of rabbit arterial blood were collected in 5 cc. oxalate. To separate portions of 10 cc. each were added respectively, 10 cc. of water, 10 cc. aqueous solutions of ammonium sulphate. Sodium tungstate and sulphuric acid were added in the usual manner. The ammonia contents of the centrifugates were estimated by two different methods (*a*) by distillation, (*b*) by the Nash-Benedict apparatus [1921].

Mg. ammonia N per 100 cc. blood.

Total ammonia found		Ammonia added	Ammonia recovered	
(*a*)	(*b*)		(*a*)	(*b*)
0·05	0·04	—	—	—
0·11	0·10	0·054	0·06	0·06
0·25	0·23	0·22	0·20	0·19
0·43	0·43	0·44	0·38	0·39

From these experiments it was concluded that if the blood samples were precipitated immediately after collection the ammonia content of the centrifugates could be estimated at leisure on the same day and that the values obtained would represent with sufficient accuracy the ammonia content of the shed blood.

The formation of ammonia in shed blood.

Having confirmed the findings of Parnas and Heller on the rapid formation of ammonia in rabbit blood *in vitro*, we were interested in the results obtained by Nash and Benedict [1921]. The evidence advanced by these authors indicated that the ammonia content of the shed blood of dogs did not increase during a post-collection period of several hours. On repeating these experiments we found the very marked specific differences shown in Fig. 1. For a comparison of methods, parallel estimations on the same blood were made

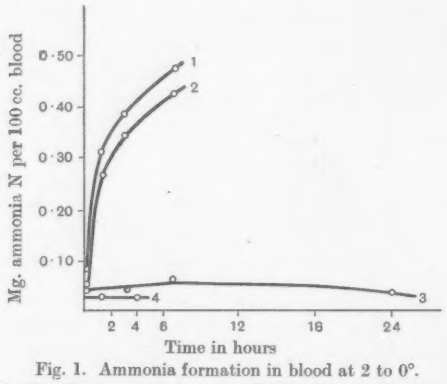

Fig. 1. Ammonia formation in blood at 2 to 0°.
1. Rabbit blood—Distillation method. 2. Rabbit blood—Nash-Benedict method.
3. Dog blood—Nash-Benedict Method. 4. Dog blood—Distillation method.

by our method and by that of Nash and Benedict. In each case the animal was bled from an artery during ether anaesthesia. The blood was collected in oxalate and was kept at – 2 to 0°.

The differences in behaviour between rabbit and dog blood are so striking as to suggest that the latter does not contain a labile ammonia-forming substance or that some necessary enzyme is absent. Incidentally the results also show that by the application of two very different methods of estimation essentially the same values for the ammonia content of the blood have been obtained. This indicates either that the two methods give accurate and absolute values or that both are subject to a common error. Of the two possibilities the latter is the more improbable.

Experiments with urea.

In Exps. 1–5 which follow we have studied the effect of an increased urea concentration in the cells of the gastric mucosa on the ammonia content of the gastric venous blood. The results have been summarised in Table I.

From these experiments it was concluded that if the urea concentration in the cells of the gastric mucosa be increased, either by the gastric absorption of urea or by diffusion from the blood, part of the urea is converted into an ammonium salt.

The administration of the urea in dilute hydrochloric acid and the positive results following intravenous injection of urea show that the observed effects cannot be due to the action of ureolytic organisms which might be in the stomach. The original experiments, which were all done *in vitro* in the presence of toluene, and in which it was found that the hydrolysis of urea was effected only by preparations of the gastric mucosa, also prove that the enzyme is a constituent of the gastric cells in the carnivora.

The results of two control experiments are presented in Table II.

The results presented in Table II, Exp. 6, proved that for the increased ammonia production in the gastric mucosa, as recorded in Table I, the presence of urea was necessary. In Exp. 7 the ammonia content of the mesenteric blood prior to the urea absorption was already so high that the results are little more than suggestive. The absence of any increase in the ammonia content, and in fact its very noticeable decrease, do at least suggest that the intestinal absorption of urea is not accompanied by an increased ammonia production. This view is supported by the results of experimentation *in vitro* by which it was shown that urea is not hydrolysed by preparations of the intestinal mucosa [Luck, 1924]. One should also refer to the persistently low values of the arterial ammonia, for they indicate the absence of any general systemic production of ammonia.

It might therefore be concluded that under the conditions of these experiments urea was converted into ammonia by the gastric mucosa only, and that this hydrolysis of urea was catalysed by gastric urease.

Table I.

	Time	Mg. N per 100 cc. blood. Portal vein NH_3	Portal vein Urea	Femoral artery NH_3	Femoral artery Urea	Remarks
Exp. 1.	Male dog of 12 kg.					
	10.30	0·17	—	—	—	
	10.36	—	—	0·08	—	
	10.40	—	—	—	—	3 g. urea in 50 cc. water administered by stomach tube
	11.12	—	—	0·10	—	
	11.30	0·27	—	—	—	
	12.10	0·52	—	—	—	
	12.15	—	—	0·12	—	
Exp. 2.	Male dog of 23 kg.					
	11.00	—	—	—	7·5	
	11.04	0·13	5·9	—	—	
	11.07	—	—	—	—	5 g. urea in 100 cc. water administered by stomach tube
	11.35	—	—	0·12	8·8	
	11.40	0·39	10·0	—	—	
	12.41	—	—	0·22	11·0	
	12.43	1·60	14·2	—	—	
	12.50	—	—	—	—	Stomach excised. Urease present in mucosa
Exp. 3.	Female dog of 9 kg. Animal pregnant. Uterus excised.					
	10.42	—	—	0·24	5·6	
	10.46	0·25	5·8	—	—	
	10.50	—	—	—	—	3·7 g. urea in 75 cc. 0·05 *N* HCl by stomach tube
	11.42	0·54	13·6	—	—	
	11.45	—	—	0·21	12·3	
Exp. 4.	Male dog of 13 kg.					
	10.55	—	—	0·07	12·5	
	11.00	0·07	12·3	—	—	
	11.04	—	—	—	—	5 g. urea in 100 cc. 0·05 *N* HCl by stomach tube
	11.47	—	—	—	17·8	
	11.50	0·12	17·0	—	—	
	1.04	—	—	0·05	18·1	
	1.06	0·28	26·2	—	—	
Exp. 5.	Male dog of 15 kg.					
	10.31	—	—	0·07	—	
	10.40	0·13	—	—	—	
	10.50–11.10	—	—	—	—	4 g. urea in 100 cc. 0·085 % NaCl injected in the left femoral vein
	11.50	0·14	—	—	—	
	12.53	—	—	0·11	—	
	12.56	0·32	—	—	—	
	1.10	—	—	—	—	Stomach excised. Urease present in the mucosa

(Note to Exp. 5.—To induce acidosis, the animal received on each of the two fasting days 4·8 g. anhydrous calcium chloride in milk.)

Experiments with ammonium salts.

Though we have shown that gastric urease functions *in vivo* by the hydrolysis of urea it is also apparent that it may be capable of urea synthesis. In Exps. 8 and 9 we have followed the urea content of arterial and gastric venous blood during the gastric absorption of ammonium salts.

Table II.

	Time	Mg. ammonia N per 100 cc. blood. Portal vein	Femoral artery	Remarks
Exp. 6.	Male dog of 13 kg.			
	10.52	—	0·19	
	10.55	0·18	—	
	11.00	—	—	100 cc. 0·05 *N* HCl by stomach tube
	12.04	0·15	—	
	12.56	—	0·14	
	1.03	0·18	—	
Exp. 7.	Female dog of 18 kg.			
	10.20	—	0·02	
	10·30	1·39	—	
	10·45	—	—	4 g. urea in 100 cc. 0·05 *N* HCl were injected
	11.23	—	0·06	into a loop of the small intestine (jejunum and
	11.28	0·49	—	ileum)
	12.26	0·46	—	

(Note to Exp. 7.—The spleen was tied off and the splenic vein was ligatured close to the portal vein. The mesenteric vessels were not tied.)

Table III.

		Mg. N per 100 cc. blood. Portal v.		L. gastro-epiploic v.		Femoral a.		Femoral v.	
	Time	NH_3	Urea	NH_3	Urea	NH_3	Urea	NH_3	Urea
Exp. 8.	Male dog of 12 kg.								
	10.30	—	—	0·20	8·5	—	—	—	—
	11.05	—	—	9·8	8·2	—	—	—	—
	11.13	—	—	—	—	—	—	—	10·6
	11.16	—	—	—	—	2·10	—	—	—
	12.03	—	—	2·0	14·9	—	—	—	—
	12.09	—	—	—	—	—	—	0·98	13·1
	12.10	—	—	—	—	1·02	17·3	—	—
	12.15	2·29	16·7	—	—	—	—	—	—
	12.47	—	—	3·2	19·4	—	—	—	—
	12.52	—	—	—	—	—	—	1·1	12·8
	12.54	—	—	—	—	1·01	17·9	—	—
	12.57	3·4	19·4	—	—	—	—	—	—

(Note.—10.34: 5 g. ammonium chloride in 100 cc. water by stomach tube.)

Exp. 9.	Male dog of 11 kg.								
	10.59	—	—	—	—	0·11	—	—	—
	11.02	0·21	13·4	—	—	—	—	—	—
	11.50	—	—	—	—	0·36	19·4	—	—
	11.55	3·9	19·2	—	—	—	—	—	—
	12.43	—	—	—	—	0·35	23·1	—	—
	12.48	2·5	21·5	—	—	—	—	—	—

(Note.—On each of the two fasting days the animal received 10 g. sodium bicarbonate in milk. 11.08: 4 g. ammonium carbonate in 100 cc. water by stomach tube.)

As the urea content of the blood leaving the stomach is practically the same as that of the inflowing arterial blood, there is no indication of any gastric synthesis of urea from the absorbed ammonia. The available evidence therefore favours the view that gastric urease functions *in vivo* by the conversion of urea into ammonium carbonate and that to no measurable degree does it manifest any synthetic function.

Discussion.

As a first consideration it is apparent that gastric urease cannot be associated with the production of the urinary or blood ammonia. Any ammonia which it may produce under the most favourable conditions would be promptly converted into urea by the liver. Even if some general significance could be attached to the portal ammonia, most of it enters from the mesenteric veins and not from the gastric veins.

As a second conclusion, it would therefore follow that gastric urease could have only a local function if any. This consideration limits the function of the enzyme to the production of ammonia for secretion into the stomach or in response to some local intracellular requirement. Without becoming involved in too much speculation it is sufficient at present to enumerate the various possible functions of the enzyme that have been suggested to us, viz., the secretion of alkali into the stomach for the control of gastric hyperacidity [McBaird, Campbell and Hern, 1924; Huber, 1917], an intracellular mechanism for the protection of the cells of the mucosa against the highly acid gastric juice with which they are in contact, a mechanism for the local regulation of the movements of the stomach wall either by the direct effect of the base (ammonium carbonate) on the muscle fibres [Kupaloff, 1924; Goldenberg, 1924] or as a specific property of ammonium ions [Práwdicz-Neminski, 1924]. A final suggestion would be to class the enzyme as a vestigial structure. Without the support of further experimental evidence we are not prepared however to ascribe any specific function to the enzyme.

Summary.

(1) Under normal physiological conditions urea constantly diffuses into those cells of the gastric mucosa which contain urease and is converted into ammonia. In dogs under ether anaesthesia an increased diffusion of urea caused either by the gastric absorption or intravenous injection of that substance is accompanied by an increase in the ammonia content of the gastric venous blood.

(2) The gastric absorption of ammonium salts is not accompanied by an increase in the urea content of the gastric venous blood.

(3) The enzyme appears to function only by the conversion of urea into ammonia in response to local conditions or requirements.

(4) The shed blood of dogs shows no increased ammonia content in 24 hours at -2 to $0°$. That of rabbits rapidly increases.

We would conclude by expressing our indebtedness to Sir F. G. Hopkins for much helpful criticism, to Dr J. B. S. Haldane for many valuable suggestions, and to the Medical Research Council for the assistance that has been extended to one of us (J. M. L.).

Note. Since preparing these results for publication an interesting paper by Parnas [1925] has appeared in which are reported the blood ammonia values for several different species. His observations on the specific differences in the behaviour of rabbit and dog blood after long standing are in entire agreement with the results which we have recorded.

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LV. THE RELATION BETWEEN THE METABOLISM AND THE SPECIFIC DYNAMIC ACTION OF AMINO-ACIDS.

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In a series of papers on "Animal Calorimetry," by Lusk and his collaborators [1912–1924], the view has been put forward that the specific dynamic action of proteins is due to a chemical stimulation of the body cells to a higher level of metabolism by some intermediary metabolites of the amino-acids other than the immediate products of their deamination. Accordingly one would expect, *a priori*, the amino-acids glycine and alanine—which exert a profound specific dynamic action—to be metabolised in the organism more rapidly and more completely than leucine, aspartic acid, etc., which are known to exert no appreciable specific dynamic action [Lusk, 1915; Atkinson and Lusk, 1918]. In other words, one would expect a smaller increase in the amino-N content of blood and a quicker return of the same to the normal level, following the ingestion of glycine or alanine, than after the ingestion of an equivalent amount of leucine or aspartic acid.

However, contrary to expectations based on Lusk's theory, the observations of Bang [1916, 2] show that whereas the amino-acid content of rabbit's blood increases very rapidly after an oral administration of glycine or alanine, and remains at a high level for about 6 hours, the ingestion of leucine causes no significant increase at all in the amino-acid content of blood.

In view of the importance and interest of the problem and the apparent inconsistencies existing between Lusk's theory and Bang's observations, it was decided to repeat Bang's experiments on a more extended scale. The results of this part of the investigation are described in Part I.

Further, having recently been impressed by the undesirability of applying facts based on observations on one species of animals to other species, we extended Bang's experiments to dogs in order to make sure that the vastly different points of view arising from the work of Lusk and Bang were not due to some intrinsic metabolic differences in the species of animals used by them. The modified procedure adopted in these experiments was designed firstly to eliminate any effect due to variable times of passage of the ingested material

through the pylorus, and secondly to enable us to follow the course of intestinal absorption more closely by analysing blood samples from the portal vein. This part of the investigation is dealt with in Part II.

A third series of experiments in which the amino-acids were administered to dogs by intravenous injection is described in Part III. These experiments were designed for the purpose of eliminating any effect due to variable rates of absorption from the intestine.

Experimental.

PART I

Rabbits of approximately uniform size were employed, each animal being used not more frequently than once a fortnight, during which interval it was kept on the ordinary routine diet. For 48 hours before the experiment the rabbit was kept in a clean metabolism cage and was given only water. On the experimental day, blood samples of about 1·5 cc. each were obtained from the ear at suitable intervals, and received into 0·15 cc. ammonia-free 2 % potassium oxalate solution. After drawing the first sample of blood a neutralised solution, at 38°, of the substance under investigation was administered by stomach tube. The total volume of the fluid ingested was 100 cc., and contained 560 mg. of amino-N (equivalent to 3 g. glycine).

Immediately after drawing each sample, the blood proteins were precipitated by the method of Folin and Wu [1919]. For the estimation of urea, 0·3 cc. molar phosphate solution of p_H 7·45, and 1 cc. of 0·5 % urease [Van Slyke and Cullen, 1914] filtered through glass-wool, were added to 4 cc. of the protein-free blood filtrate, and, after standing for 45 minutes at 40°, the ammonia was distilled (using 2 cc. saturated borax solution) into standard acid, and estimated by nesslerisation.

The amino-N was estimated in the blood filtrate by Folin's colorimetric method [Folin, 1923] which yielded very consistent and satisfactory results.

The water and other reagents used were ammonia-free, and all apparatus was washed with such water.

The amino-acids investigated were glycine, alanine, leucine, aspartic acid, glutamic acid, cystine, histidine and tryptophan, of which only the first three were used by Bang [1916, 2].

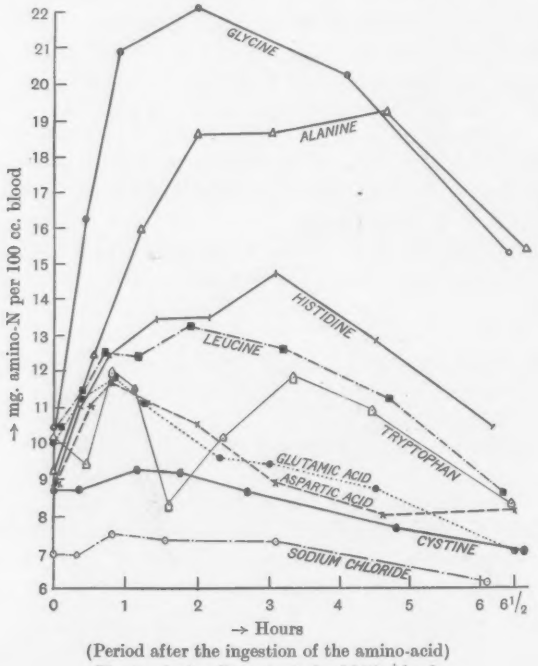
Results.

Table I shows the changes in the urea content of rabbit's blood during a period of about 7 hours following the ingestion of the various amino-acids, and Fig 1[1] represents the corresponding changes in the amino-acid content of the blood, under the same conditions.

[1] In the case of tryptophan the amino-N content of blood was reckoned as the difference between the total-non-protein N and urea-N, and the figures thus obtained being rather high, only two-thirds of their actual values were plotted in Fig. 1 for the sake of a clearer comparison.

Table I. *The Urea-values in this Table are expressed in mg. urea-N per 100 g. blood.*

Substance under investigation	Period after ingestion of amino-acid							Difference between initial and maximum urea values	Remarks
	0 min.	20–25 mins.	45–50 mins.	75–90 mins.	2½–3 hrs.	4–4½ hrs.	6–6½ hrs.		
Glutamic acid (6·0 g.)	9·6	10·3	10·9	12·6	14·7	14·8	18·6	9·0	
Glycine (3·0 g.)	18·0	—	19·6	17·6	19·1	22·2	26·7	8·7	
Aspartic acid (5·3 g.)	13·6	13·3	15·8	17·2	19·9	20·1	21·7	8·1	
l-Alanine (Kahlbaum) (3·6 g.)	15·8	—	—	18·8	23·4	23·4	16·4	7·6	
Cystine (4·8 g.)	12·4	12·4	—	17·9	18·7	14·0	15·1	6·3	Administered as the sodium salt
Tryptophan (4·5 g.)	14·3	16·1	16·6	18·3	20·0	19·4	18·2	5·7	Animal had acute diarrhoea about 1 hour after ingestion of tryptophan
l-Leucine (Kahlbaum) (5·2 g.)	14·5	14·4	15·7	18·3	17·5	17·5	18·8	4·3	Administered as the sodium salt
Histidine hydrochloride (8·3 g.)	21·6	22·9	22·3	22·0	19·3	21·2	21·7	1·3	
Histidine hydrochloride (repeated) (8·3 g.)	18·9	20·1	20·0	20·1	18·9	18·4	19·0	1·2	
Sodium chloride (1·2 g.)	10·7	8·5	8·8	9·6	10·1	—	6·1	−0·6	Control experiment



(Period after the ingestion of the amino-acid)

Fig. 1. Amino-N content of rabbit's blood.

These results (Table I) indicate that whereas there is no marked difference in the extent of urea formation in the case of glycine, alanine, aspartic acid and glutamic acid, there is a progressive decrease in the case of cystine, tryptophan, leucine and histidine, the last one producing practically no increase in the blood urea during the entire experimental period of 6 hours. On the other hand, the amino-acid content of blood (Fig. 1) after the ingestion of glycine, alanine or histidine not only attains a very high maximum, but persists at a high level all through the experimental period; whereas the amino-acid content of blood in the case of the other amino-acids examined, without ever rising very high, comes down to the normal or subnormal level within 3–6 hours, in confirmation of Bang's observations [1916, 2]. The significance of these results is discussed elsewhere.

PART II

Having in the main confirmed Bang's observations on rabbits regarding the amino-acid content of blood after the ingestion of glycine, alanine and leucine, and having further extended the investigation to other amino-acids, the experiments were repeated on dogs, in the following way:

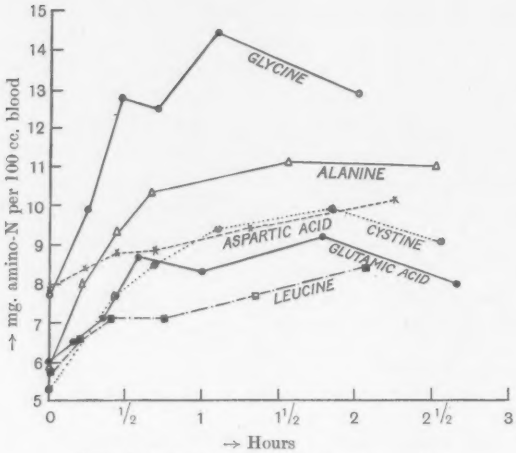
Dogs weighing about 10 kg. each were used, and they were given water only for 48 hours before use to ensure freedom of the small intestine from food. The animals were anaesthetised with ether[1]. A tracheal cannula was inserted. The femoral artery on one side and the femoral vein on the other were exposed. The abdomen was then opened and the splenic vein was tied off close to the portal vein. Ligatures were then applied to the small intestine, one just below the duodenum and the other above the caecum. The femoral artery was then ligatured distal to the profunda artery. Blood samples were taken by syringe at suitable intervals from the femoral artery and vein and from the portal vein, the latter being punctured about 2–3 inches from the porta hepatis. After drawing the first set of blood samples, the substance under investigation in a neutralised solution[2] at 38°, was injected into the small intestine by means of a hypodermic needle and pipette. The abdomen was promptly closed with artery clips. The total volume of the fluid injected was 100 cc. The animal was kept warm throughout the experiment.

The collection of blood samples (about 1·5 cc. each), the removal of blood proteins and the estimation of urea and amino-N in the blood filtrates, were done exactly as described before (p. 367).

The amino-acids investigated were glycine, alanine, leucine, aspartic acid, glutamic acid and cystine, the amounts used being the same as in the experiments on rabbits (see Table I).

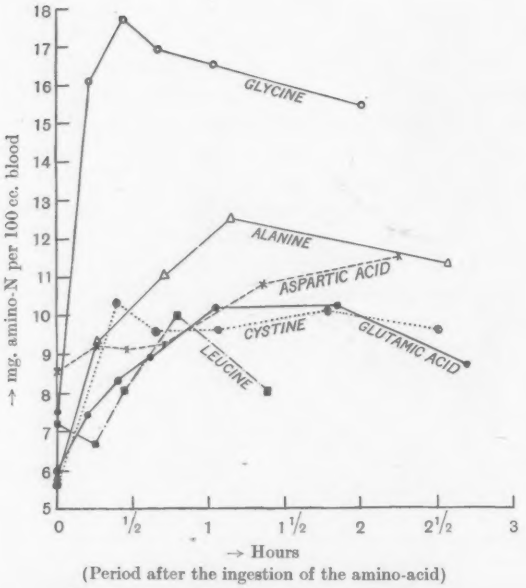
[1] Ether anaesthesia has, according to Okada and Hayashi [1922] no significant effect on the amino-acid content of dog's blood, during the experimental period of about 2 hours.

[2] Cystine was administered as the sodium salt, whilst the leucine solution was neutralised just before injection.



(Period after the ingestion of the amino-acid)

Fig. 2. Amino-N content of dog's arterial blood.



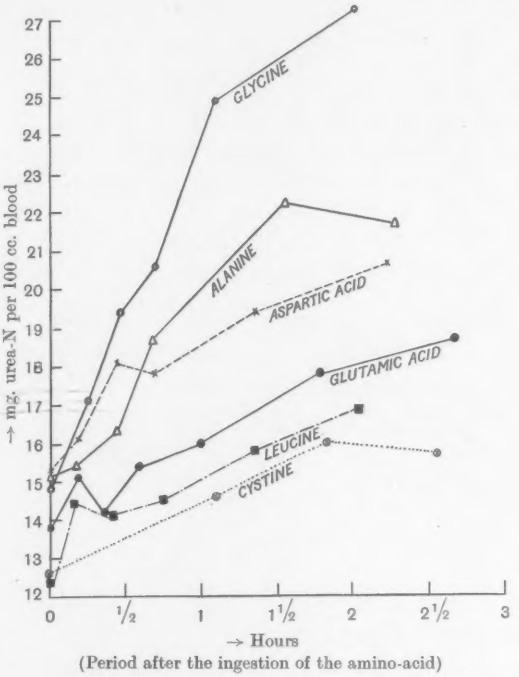
(Period after the ingestion of the amino-acid)

Fig. 3. Amino-N content of dog's portal blood.

Results.

(i) In confirmation of the observations made on rabbits (see Part I), Figs. 2 and 3 show that the amino-acid content of blood after the ingestion of glycine or alanine not only attains a very high maximum but persists at a high level all through the experimental period, whereas in the case of the other amino-acids studied, the amino-N content remains at a comparatively low level throughout. The possible significance of this observation is discussed later.

(ii) The extent of urea formation in the case of glycine and alanine is higher than in the other cases (Fig. 4).



(Period after the ingestion of the amino-acid)

Fig 4. Urea content of dog's arterial blood.

(iii) A comparison of Figs. 2 and 3 shows that at any one time the amino-acid value of portal blood is higher than the corresponding arterial value. This presumably results from the dilution of the portal blood with systemic blood. In the early stages of the experiment, the amino-N content of femoral-venous-blood[1] is also lower than that of the corresponding arterial sample. Later

[1] The figures for amino-N of femoral-venous blood were obtained in the case of glycine, alanine and cystine only, but they are not included in Fig. 2, in order to avoid unnecessary complication of the curves.

on, the two values tend to be almost identical. These observations, supported by the results of our injection experiments (Part III), point to a rapid absorption of the amino-acids from blood by the tissues. These conclusions are in agreement with similar findings of Folin and Denis [1912, 1] and of Van Slyke and Meyer [1912].

PART III

Dogs weighing about 10 kg. each were used. After being given only water for 48 hours, the animals were anaesthetised as described in Part II. The femoral artery on one side and the femoral vein on the other were exposed. The bladder was emptied by pressure. The femoral artery was then ligatured distal to the profunda artery. After drawing the first arterial blood sample by syringe, as described in Part II, the amino-acid under investigation, dissolved in 0·85 % NaCl solution, neutralised to litmus and warmed to 38°, was injected into the femoral vein as rapidly as possible (usually in less than a minute). Subsequent arterial samples were drawn at suitable intervals and analysed for amino-N and urea, as described in Part II. The total volume of the fluid injected was 25 cc., and it contained 186·6 mg. of amino-N. The amino-acids investigated were glycine, alanine, and aspartic acid.

At the end of the experimental period, the bladder was emptied and washed by catheterisation, the urine being subsequently analysed for amino-N.

Table II. 1·0 *g. glycine injected intravenously.*

Intervals before or after injection	mg. N per 100 cc. blood	
	Amino-N	Urea-N
Before injection	5·9	7·7
2 mins. after injection	10·0	7·9
8 " "	8·4	7·8
17 " "	6·7	8·7
28 " "	6·5	8·2
38 " "	6·3	8·7
57 " "	5·8	7·6
69 " "	5·8	7·7
94 " "	5·9	8·3
122 " "	5·4	8·5

Essentially similar results were obtained with alanine and aspartic acid.

These results (Table II) show that:

(i) Immediately after injection, the amino-acid content of the blood falls very rapidly, reaching almost the normal level within 15 minutes.

(ii) During the experimental period of 2 hours, there is no significant increase in the blood urea.

No measurable amount of amino-N was eliminated in the urine during the course of the experiment.

From these observations it is concluded that, under the conditions of our experiments, the tissues absorb the amino-acids almost completely and with extreme rapidity from the blood, and that the formation of urea follows much later.

Discussion.

We have shown (Parts I and II) that the extent of deamination of glycine and alanine is, in the case of rabbits of the same order, and in the case of dogs even greater than that of the other amino-acids studied. Again our injection experiments (Part III) have shown that under similar conditions the rate of absorption of the different amino-acids by the tissues is of a similar order, and also that the excretion of the free amino-acid is negligible under the conditions of our experiments. From these considerations it is clear that the earlier and greater increase in the blood amino-N, and its persistent circulation at a high level following the ingestion of glycine or alanine (both in rabbits and in dogs), is due neither to the lack of deamination nor to the non-absorption of these amino-acids by the tissues.

It is suggested that this phenomenon is due to the rapid absorption of glycine and alanine from the intestinal tract, as shown by the early and rapid rise in the amino-N of the portal blood, and its persistence at a high level for a long time (Fig. 3). Levene and Kober [1909] and Levene and Meyer [1909] have shown that glycine, alanine, glycyl-glycine and aspartic acid are rapidly metabolised, whilst leucine, phenylalanine, arginine and asparagine are metabolised more slowly. They ascribe these differences to the different rates of absorption of these amino-acids from the alimentary canal. Further, the work of Bang [1916, 1] and of Lewis [1918], on the relation between the amount of amino-acid ingested and the rise in the amino-N content of blood, lends additional support to the view expressed above.

Other contributory factors leading to this phenomenon of the rapid and continuous entrance into the systemic circulation of glycine and alanine (as distinct from other amino-acids), may be firstly, the permeability of the liver cells to glycine and the non-permeability to leucine, as observed by Bang [1916, 2], and secondly, the greater adsorbability of glycine and alanine by the blood corpuscles as suggested by the work of Sbarsky and Muchamedoff [1925] on the adsorption of glycine, alanine, leucine and tyrosine by an "erythrocyte-emulsion."

From the above-stated considerations it is obvious that Bang's observations [1916, 2] regarding the persistent circulation in blood of amino-N at a high level, following the ingestion of glycine or alanine, are not necessarily out of harmony with the views of Lusk on the cause of the specific dynamic action of these amino-acids. These two apparently inconsistent points of view are reconciled and explained by the difference in the rates at which the various amino-acids enter the circulation from the digestive tract.

It may be mentioned that the greater urea production in the case of glycine and alanine (Part II) is also probably a direct result of the circulation of larger amounts of these amino-acids and their consequent higher "partial pressure" in the sense of Folin and Denis [1912, 2].

The cause of the specific dynamic action of amino-acids.

Lusk [1915] considered that the increased heat production following the ingestion of glycine or alanine is not due to the oxidation of these amino-acids or of their metabolites, but that it is caused by the chemical stimulation of the body cells to a higher level of metabolism. In their attempts to discover the nature of this stimulant and the mechanism of its action, Lusk and his associates came to the following conclusions:

(i) That the specific dynamic action of amino-acids bears no relationship to the rate or extent of deamination, as shown by the facts that the period of maximum heat production [Lusk, 1912; Csonka, 1915] precedes the period of maximum nitrogen excretion [Janney, 1915] and that glutamic and aspartic acids and asparagine, though deaminised, exert no specific dynamic action [Atkinson and Lusk, 1918].

(ii) That acid radicals as such, or hydroxy- or keto-acids, which may possibly be the intermediary metabolites of some amino-acids, do not exert any specific dynamic action [Lusk, 1921; Atkinson and Lusk, 1918].

These results left the main problem still unsolved, namely, whether the specific dynamic action is due to a stimulus provided by the free amino-acid itself or by one or more of its yet unknown non-nitrogenous metabolites. Lusk [1915], however, subscribed to the latter view and the two chief arguments which he put forward in support of his theory need some attention.

Firstly, Lusk argued that if the free amino-acids exert any specific dynamic action, one should be able to observe an appreciable accumulation of amino-acids in the tissues following the ingestion of meat. Since Wishart [1915] failed to observe any such accumulation, Lusk concluded that the stimulus to increased heat production was not provided by the free amino-acids themselves. It appears to us, however, that this argument, besides being based on observations which are contradicted by Folin and Denis [1912, 2], Van Slyke [1914] and Mitchell [1918], is in itself not very conclusive. Our observations and those of numerous other workers have established the fact that following the ingestion of a protein or an amino-acid diet, the amino-N content of blood remains at a high level for a considerable period, in spite of the rapid absorption by the tissues. This fact indicates clearly that, under the conditions described, a fresh and continuous supply of amino-N reaches the tissues during a period of several hours. Therefore, even though there may not be a static accumulation of amino-acids in the tissues, there is no reason to assume that the abundant supply of constantly circulating amino-acids is not capable of stimulating the tissue cells to a higher level of metabolism.

The second argument put forward by Lusk in support of his theory is based mainly on the observations of Janney and Csonka. Janney [1915] showed that in phlorhizinised dogs the excretion of glucose, following the ingestion of a protein, was proportional to the amount of glucogenetic amino-acid radicals present in that protein, and that the glucose excretion attained

a maximum value during the second and third hours. Csonka [1915] observed that in phlorhizinised dogs the period of maximum heat production (second and third hours) after the ingestion of meat or glycine, corresponds to the period of maximum metabolism of the ingested amino-acids, as determined by the "extra-glucose" excretion method. From these observations Lusk concluded that the specific dynamic action of amino-acids was dependent upon and proportional to their metabolism. However, this conclusion is not justified in view of the facts that glutamic and aspartic acids, in spite of their glucogenetic properties [Janney, 1915], do not exert any specific dynamic action [Atkinson and Lusk, 1918], whilst leucine and tyrosine, though non-glucogenetic [Janney, 1915], cause a certain increase in heat production [Lusk, 1915]. Again, alanine produces only about half as much "extra heat" as an iso-glucogenetic quantity of glycine [Lusk and Richie, 1915], though, according to the "glucogenesis" theory, the two should produce the same effect.

And finally, the correspondence in the periods of maximum heat production and maximum glucose excretion cannot be brought forward as an argument in support of Lusk's theory, because it has been shown by us (Fig. 1) and by Bang [1916, 2] that, under similar conditions, the amino-N content of blood is also at its maximum level during the second and third hours[1]. Hence the only conclusion that can be drawn from the above considerations is that the specific dynamic action may be due to some property of either the free amino-acid itself or its metabolites. The existing data do not preclude either of the two possibilities.

In this connection, however, the observations of Sandiford, Boothby and Giffin [1923], that "In all instances (of myelogenous leucaemia) in which amino-acids (of blood) were increased, there was some, but not necessarily a proportional increase in basal metabolism," are of interest. Similar observations on the association of pathological hyperamino-acidaemia with increased basal metabolism, have been recorded in cases of phosphorus poisoning [Atkinson and Lusk, 1918], and pernicious anaemia [Kraus, 1893; Magnus Levy, 1906; and Meyer and Du Bois, 1916]. But although an elevated basal metabolism, under physiological as well as pathological conditions, has been shown to be intimately associated with an increased concentration of amino-acids in the blood, it cannot be stated at this stage that the increased heat production is a direct consequence of hyperamino-acidaemia.

Summary.

(1) Bang's observations on rabbits regarding the persistent circulation of amino-N in the blood at a high level, following the ingestion of glycine or alanine, and the absence of an appreciable increase in the amino-N content of the blood after the ingestion of leucine, are confirmed and extended to other amino-acids, using both rabbits and dogs.

[1] In the experiments on dogs, the maximum level of amino-N in the blood is reached a little earlier (Fig. 2) owing to more rapid absorption from the small intestine.

(2) In the case of histidine, although a high and sustained level of amino-N in the blood is maintained, as in the case of glycine and alanine, yet there is no increase in the urea content of blood even after 6 hours. All the other amino-acids studied caused appreciable urea production.

(3) The specific dynamic action of an amino-acid is proportional to its power of increasing the amino-N content of the blood, following the gastro-intestinal absorption of the amino-acid.

(4) An explanation is offered for the apparent inconsistencies in the views of Lusk regarding the cause of the specific dynamic action of amino-acids and the above observations of Bang and ourselves.

(5) The cause of the specific dynamic action of amino-acids is discussed.

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LVI. ON THE METHODS OF FITTING THE FORMULA OF MICHAELIS IN RELATION TO THE EFFECT OF HYDROGEN ION CONCENTRATION ON ENZYME ACTION TO THE DATA: WITH SOME DISCUSSION OF THE RESULTS.

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I PROPOSE in this paper to discuss the theory of Michaelis regarding enzyme action and to give a note on the methods which I have found useful in fitting his theoretical curves to the experimental data. According to this theory, when an enzyme acts in solutions of different hydrogen ion concentrations only that portion of the enzyme which is not ionised retains its activity. The curve describing the rate of action of an enzyme is thus according to Michaelis: (1) "the undissociation curve of an acid," or (2) "the undissociation curve of a base," or (3) "the undissociation curve of an ampholyte." "We can therefore understand all the phenomena completely by the following interpretation: the enzyme represents an acid, a base, or an amphoteric electrolyte." Caution, however, may be needed in interpreting the experiments. With some enzymes which are not really ampholytes, the observations give a grouping which suggests this, because a strong acid or a strong alkali causes destruction of the enzyme, the diminution in action observed being due to this and not to an increased ionisation.

The physico-chemical theory and the equations formed by Michaelis are as follows. The dissociation of water into ions obeys the well-known equation:

$$H_2O \rightleftarrows H^{\cdot}+OH'$$

which is commonly written

$$[H^{\cdot}]\cdot[OH'] = k_w \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1),$$

where k_w is defined as the dissociation constant of water and is equal to 10^{-14} at temperature 18°. As $[H^{\cdot}]$ is equal to $[OH']$, the number of hydrogen and hydroxyl ions present in a neutral solution is thus 10^{-7} g. ions per litre.

With an acid enzyme, the undissociated residue, *i.e.* the ratio of the undissociated acid to the total acid denoted by ζ is given by the equation

$$\zeta = \frac{1}{1+\frac{k}{[H^{\cdot}]}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2).$$

For a base in a similar way

$$\zeta = \frac{1}{1+\frac{k}{[OH^*]}} \quad \text{.....(3).}$$

Working with such small numbers as 10^{-7}, it is convenient to use the index to denote the hydrogen ion concentration. The symbol p_H which is equal to $-\log_{10} H^*$ has been selected for convenience, p_H being a contraction for power of the radix 10. If this substitution is made in equation (2), we have

$$\zeta = \frac{1}{1+k\,10^{-p_H}} \quad \text{.....(4),}$$

or in ordinary coordinates,

$$y = \frac{1}{1+k\,10^{-x}} \quad \text{.....(5).}$$

In the case of the amphoteric electrolyte the equation becomes

$$\zeta = \frac{1}{1+\frac{k_a}{[H^*]}+\frac{k_b}{k_w}[H^*]} \quad \text{.....(6),}$$

where k_a and k_b are the dissociation constants of the acid and the base. This equation describes a symmetrical curve. Referring to the axis of symmetry it becomes

$$\zeta = \frac{1}{1+k\,(10^{\xi}+10^{-\xi})} \quad \text{.....(7),}$$

which is the convenient form for fitting. If ϕ is the distance of the axis of symmetry from the origin in (6)

$$k_a = k\,10^{\phi}$$

and

$$k_b = k_w\,k\,10^{-\phi}.$$

In the case of a dibasic acid

$$\zeta = \frac{1}{1+\frac{k_1}{[H^*]}+\frac{k_1k_2}{[H^*]^2}}$$

$$= \frac{1}{1+k_1 10^{+x}+k_1k_2 10^{+2x}} \quad \text{.....(8),}$$

where k_1 signifies the first and k_2 the second dissociation constant of the acid.

In the case of an amphoteric electrolyte which is doubly dissociated both as regards the acid and basic radicals, a case not given by Michaelis

$$\zeta = \frac{1}{1+k_1\,(10^{\xi}+10^{-\xi})+k_2\,(10^{+2\xi}+10^{-2\xi})} \quad \text{.....(9).}$$

k_1 and k_2 are here constants such as k in equation (7).

Cor. It may happen that double dissociation is confined either to the acid radical or the basic in which case the term $10^{2\xi}$ or $10^{-2\xi}$ is omitted respectively.

A further case not considered by Michaelis is that in which the dissociation takes place from grouped molecules. In this case ξ is replaced by $c\xi$, and the curve of the amphoteric electrolyte, equation (7), becomes

$$\zeta = \frac{1}{1+k\,(10^{c\xi}+10^{-c\xi})} \quad \text{.....(10).}$$

A considerable number of enzymes have been examined and the agreement of the theory and experiment is in many cases very close. The lipase of the stomach may be taken as an example of an acid enzyme, and that of the pancreas of an alkaline, as shown by the experiments of Davidsohn [1913]. The examples of ampholytes are numerous and it is only necessary to refer to the experiments by Sörensen [1909] with catalase where observations and the theory are in very close accord. There are, however, many exceptions. The inversion of raffinose by the enzyme raffinase is one case. The simple theory no longer accounts for the phenomena observed. In the accompanying diagram (Diagram I), the observations of Kuriyama and Mendel [1917] have been graphed and two curves drawn through the observations. The chain line shows the curve of an ampholyte with one dissociation as given by Michaelis, the continuous line the curve with two dissociations. The observations are, as is obvious from the diagram, not of a high order of accuracy, but I think they are sufficiently accurate to show that the second curve accords more closely with the facts.

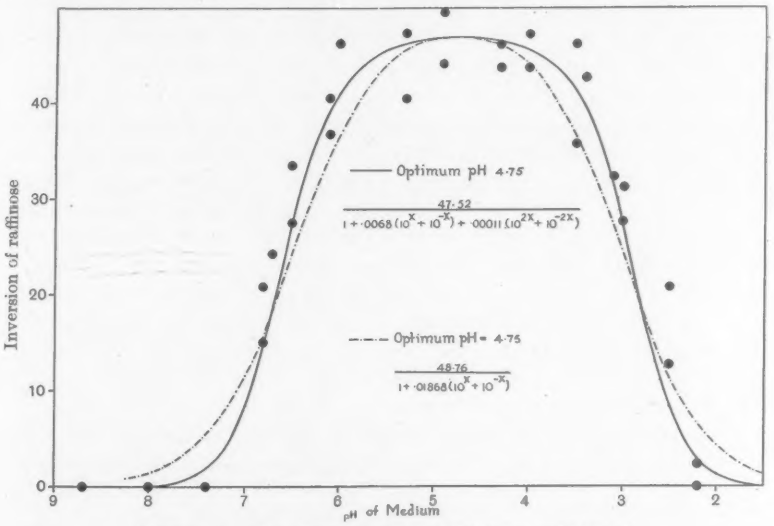

Diagram 1. Action curve of raffinose (Kuriyama and Mendel).

In testing any theory much is to be learnt from the exceptions to the theory. For instance, with regard to the observations on a certain enzyme, which observations I had permission given me to use, but which I do not wish to use at the present moment because the experiments are being repeated, the figures obtained were irreconcilable with the Michaelis theory as given but were reconcilable with that theory if the molecules of the enzyme existed on an average linked in pairs and if these pairs tended to lose one ion. In this case there may be an error but the figures are so definite that I think it

unlikely that the repetition of the experiments will alter the conclusions. In other words the formula (10) applies, in this case the value of c being equal to 0·5. To certain other observations on enzymes, the same remarks apply, but the data are not sufficient to permit of an undoubted interpretation. Data, however, which are sufficient to settle this point may be obtained from the observations on lysins.

The experiments examined are those of Walbum [1915] who worked with various haemolysins and gives a very large number of tables and diagrams illustrating the phenomena. Since, however, both alkalies and acids in moderate concentrations lake red blood corpuscles, experiments with haemolysins often do not afford a sufficient range of values to permit of curve fitting. To measure the action of the acid or of the alkali separately and subtract this effect from the total effect is also of doubtful validity as it cannot be directly inferred that a mixture of lysin and acid or alkali produces only an additive effect. The effect of a lysin or an acid or alkali may be greater upon a slightly damaged, than on a normal red blood corpuscle.

One lysin, however, that of the poison of the bee, furnishes quite sufficient data. The bloods of three animals, the sheep, the ox, and the horse, were used. The observations on ox blood corpuscles fit a Michaelis curve having an optimum action with $p_{\mathrm{H}} = 7{\cdot}6$ very well. A rather better fit is obtained on the assumption that two ions are dissociated. The observations, however, have a considerable probable error and the curves therefore cannot be statistically separated. With sheep corpuscles the dissociation of two ions is, however, quite definitely required. Both theories fail with the blood corpuscles of the horse. If the Michaelis theory holds here, only one explanation seems possible, namely, that from two linked molecules, an average of five ions is liberated. The possibility of this I leave to the chemists, but it seems to me improbable. In these observations the optimum value of the hydrogen ion concentration for the action of the lysin varies with the substrate. The difference of the optimum, passing from the sheep through the ox to the horse, is fully half a unit of concentration expressed as p_{H}.

The rate of the destruction of enzymes at different hydrogen ion concentrations follows similar laws. It is found that an enzyme is destroyed more rapidly by heat as the amount of ionisation increases according to the Michaelis theory. They add nothing to the general theory. With regard to toxins and lysins, however, there are some points of interest. Observations on the destruction of a staphylolysin have been made by Walbum [1922, 1] at three different temperatures and in a range of hydrogen ion concentrations from 4·5–9·5. The observations and the curves I have fitted are given in the accompanying table (Table I). The observations at 30° show an asymmetrical curve, the descent being slower on the alkaline side than on the acid. These are compatible with the hypothesis that there are two dissociations on the acid side and one on the alkaline. The optimum of permanence is with $p_{\mathrm{H}} = 6$. Experiments at temperatures of 35° and 40° show a different phenomenon. The

optimum concentration for survival is at the same point as before, but in this case the curves are symmetrical and the observations can only be fitted with two dissociations on both sides of the optimum. It is to be noted that with increase of temperature, the range of survival is continuously limited.

Table I. *Destruction of a staphylolysin at three different temperatures.*

30°			35°			40°		
	Amount in 1 cc.			Amount in 1 cc.			Amount in 1 cc.	
p_H	Act.	Th.	p_H	Act.	Th.	p_H	Act.	Th.
9·09	2·0	3·66	8·70	2·0	2·42			
8·65	9·52	8·25	8·32	5·71	7·37	8·04	2·0	2·41
8·16	17·5	15·97	7·85	18·2	16·77	7·45	13·3	12·77
7·55	22·2	24·11	7·20	25·0	25·51	6·37	25·6	28·58
6·44	27·0	28·40	5·95	28·6	28·59	5·28	28·6	27·10
5·33	28·6	28·09	4·83	25·0	24·95	4·51	13·3	14·26
4·54	24·4	23·75	4·15	14·7	14·77	3·89	2·0	1·93
3·91	10·4	10·73	3·75	2·0	6·85	3·59	2·63	0·84
3·61	5·26	4·54						

The same law also applies to the destruction of toxins (Diagram II). Walbum [1922, 2] gives details regarding the destruction of the diphtheria toxin with hydrogen ion concentration for two different years, namely, 1919 and 1920. The toxin prepared in 1919 shows a symmetrical curve requiring for a fit the dissociation of two ions on both sides. The curve for 1920 requires two dissociated ions on the acid side but only one on the alkaline. As the toxins were of equal efficacy this would seem to indicate that part of the complex of which the toxins are constituted is subject to variation and suggests that this part is not an essential constituent of the toxic group of atoms.

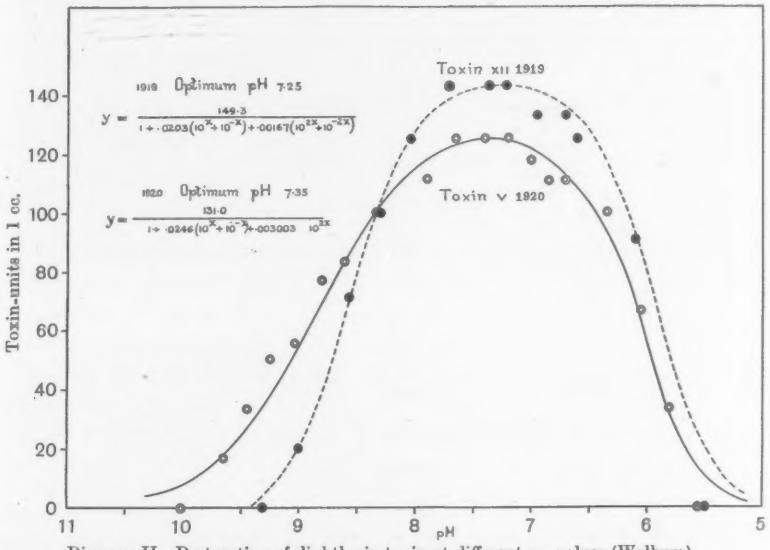

Diagram II. Destruction of diphtheria toxin at different p_H values (Walbum).

Appendix.

I. The first of the curves is that with the equation

$$y = \frac{a}{1 + k\,10^{-x}}.$$

The easiest way to fit this curve is to draw a free hand line through the observations and then taking two values of the ordinates at a distance of one unit of hydrogen ion concentration, to solve for the constants. If the two values be α and β, the solution is

$$k = \frac{\beta - \alpha}{10\alpha - \beta}.$$

The value for a is

$$a = \alpha\,(1 + k).$$

The middle point of the curve is at a distance $-\log k$ from the point of origin chosen. The rest of the curve may immediately be drawn by using the values of 10^x given in the table. Plotting the curve thus calculated through the observations the amount of readjustment can be guessed at once. By moving the middle point either to the right or to the left the process described may be repeated. No further approximation is usually required. The fit assumes that the maximum a is not known. If it be known one observation alone is sufficient to determine k.

Table II. *Table showing the values of the functions used in the calculations.*

x	10^x	10^{-x}	$10^x + 10^{-x}$	$10^{2x} + 10^{-2x}$
0	1·000000	1·0000	2·0000	2·0000
·25	1·778279	·5623	2·3406	3·4785
·50	3·162278	·3162	3·4785	10·1000
·75	5·623413	·1778	5·8012	31·6544
1·00	10·00000	·1000	10·1000	100·0100
1·25	17·78279	·0562	17·8390	316·2310
1·50	31·62278	·0316	31·6544	1000·0010
1·75	56·23413	·0178	56·2519	3162·2783
2·00	100·0000	·0100	100·0100	10000·0001
2·25	177·8279	·0056	177·8335	31622·7800
2·50	316·2278	·0032	316·2310	100000·0000
2·75	562·3413	·0017	562·3431	316227·8000
3·00	1000·000	·0010	1000·0010	1000000·0000
3·25	1778·279	·0006	1778·2796	
3·50	3162·278	·0003	3162·2783	
3·75	5623·413	·0002	5623·4132	
4·00	10000·00	·0001	10000·0001	
4·25	17782·79	·0001	17782·7901	
4·50	31622·78	·0000	31622·7800	
4·75	56234·13	·0000	56234·1300	
5·00	100000·0	·0000	100000·0000	
5·25	177827·9	·0000	177827·9000	
5·50	316227·8	·0000	316227·8000	
5·75	562341·3	·0000	562341·3000	
6·00	1000000·	·0000	1000000·0000	

II. The next curve is that referring to the amphoteric electrolyte:

$$y = \frac{a}{1 + k\,(10^x + 10^{-x})}.$$

Here it is best to determine from the observations the situation of the axis of symmetry. Having drawn a free hand curve through the graph of the data

and then ruled a series of lines parallel to the axis of x, the middle points of these lines are determined with the help of a scale and then averaged. This average may be taken as the axis of symmetry. Only two ordinates are needed to determine a and k. If α and β be the two values chosen, α referring to the ordinate at the axis of symmetry and β to that at a distance of unit hydrogen ion concentration from it, we have at once

$$k = \frac{\alpha - \beta}{10 \cdot 1\beta - 2\alpha}$$

$$a = \alpha\,(1 + 2k).$$

The curve can at once be constructed by the use of the table $10^x + 10^{-x}$. As the curve is symmetrical only one side needs to be calculated. When the calculated curve has been fitted to the observations it is quite easy to see whether a further approximation is necessary or not.

III. Sometimes the data cannot be adjusted to the formula just given; such a case is the hydrolysis of raffinose. It is necessary then to proceed to the formula describing double dissociation, *i.e.*

$$y = \frac{a}{1 + k\,(10^x + 10^{-x}) + \lambda\,(10^{2x} + 10^{-2x})}.$$

The solution of this equation is obtained as in the last case by choosing the axis of symmetry, estimating its ordinate, also the ordinate at the distance of one unit of hydrogen ion concentration from it and that also at a distance of two units. The method of solution is simply to put the appropriate values of x and y in the equation and solve the three simultaneous equations for the three constants a, k and λ. If α, β and γ be the three values at the axis of symmetry and at a distance of one and two units of hydrogen ion concentration respectively, the formulae are as follows

$$a\left\{\frac{90998 \cdot 00091}{\alpha} - \frac{19799 \cdot 9802}{\beta} + \frac{179 \cdot 82}{\gamma}\right\} = 71377 \cdot 84071,$$

$$89 \cdot 91\lambda = \left(\frac{a}{\beta} - 1\right) - 5 \cdot 05\left(\frac{a}{\alpha} - 1\right),$$

$$\tfrac{1}{2}\left(\frac{a}{\alpha} - 1\right) = k + \lambda.$$

In this case k and λ are essentially positive, if either is found to be negative the formula cannot hold. The drawback to this method is that in calculating a, at least five decimals are required. This follows because in general $\frac{a}{\alpha}$ and $\frac{a}{\beta}$ will have both of the first figures unity with a zero following, and as in the formula unity is subtracted from these ratios, it is necessary to have four significant figures.

IV. In the case where there is unequal dissociation on either the acid or the alkaline side one term in the coefficient of λ disappears. Here the formula is

$$\frac{a}{1 + k\,(10^x + 10^{-x}) + \lambda\,10^{2x}}.$$

The equations for solution are then

$$a\left\{\frac{90999}{a}-\frac{19899{\cdot}99}{\beta}+\frac{189{\cdot}9}{\gamma}\right\}=71288{\cdot}91,$$

$$100\left(\frac{a}{\alpha}-1\right)-\frac{a}{\beta}-1=189{\cdot}9\,k,$$

and

$$\frac{a}{\alpha}-2k-1=\lambda.$$

V. The last case to be considered is the one in which "linked" molecules occur. The equation is

$$y=\frac{a}{1+k\,(10^{ex}+10^{-ex})}.$$

The solution is really simple but in the process of elimination some unsuitable solutions are introduced. If 10^e be denoted by l, we have, choosing the axis of symmetry and taking three values at distances of unit hydrogen ion concentration, the following three equations

$$\alpha=\frac{a}{1+2k} \quad \text{.....(1),}$$

$$\beta=\frac{a}{1+k\,(l+l^{-1})} \quad \text{.....(2),}$$

$$\gamma=\frac{a}{1+k\,(l^2+l^{-2})} \quad \text{.....(3).}$$

These lead, after the inapplicable roots have been removed, to an equation for l of the form

$$l^2-\left\{\frac{\alpha\,(\beta-\gamma)}{\gamma\,(\alpha-\beta)}-1\right\}l+1=0 \quad \text{.....(4).}$$

The roots of this equation have the values l and $\frac{1}{l}$. From equations (1) and (2), we have

$$k=\frac{\alpha-\beta}{p\beta-2\alpha},$$

where p denotes $l+\frac{1}{l}$ or the coefficient of l in equation (4). From equation (1), a is at once obtained. For interpolating values, between those observations originally chosen for fitting, if these are required for graphical purposes, it is usually sufficient to take the values of the square roots of l and $\frac{1}{l}$ from a table of square roots and the values of l^2 and l^{-2} from a table of squares, whence $l^{1{\cdot}5}$, $l^{2{\cdot}5}$, etc. can be immediately calculated.

With lysins the range of activity is often less than with enzymes, in which case it may be inconvenient to take the unit hydrogen ion concentration as the basis of measurement, half a unit or even less being more convenient. This does not change the solution given above but only the numerical interpretation.

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LVII. A STUDY OF THE OXIDATION OF THE AMMONIUM SALTS OF NORMAL SATURATED FATTY ACIDS AND ITS BIOLOGICAL SIGNIFICANCE.

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In a number of papers Dakin [1908, 1, 2, 3; 1910] described the production of β-keto-acids and the ketones arising from them by loss of carbon dioxide, in a series of experiments on the oxidation of the ammonium salts of normal saturated fatty acids with hydrogen peroxide. The reaction is a general one and at the time it was discovered was of great significance because it afforded great support to the evidence for the theory of β oxidation of fatty acids propounded by Knoop [1904]. It is a good instance of a process taking place *in vitro* which has helped to throw light on a similar process occurring in the living body. The reactions which occur when fatty acids and certain of their phenyl derivatives undergo oxidation in the body are not simple. Phenyl-propionic acid has been shown by Dakin [1908, 4, 5] to yield, in addition to the corresponding β-keto-acid, the β-hydroxy-acid and the α, β-unsaturated acid. It seemed worth while therefore to find out whether Dakin's observations on the oxidation of fatty acids with hydrogen peroxide could be extended to include these other oxidation products, and especially the unsaturated acids which are sometimes formed during oxidation in living animals.

Using conditions similar to but not identical with those of Dakin, attempts were made in the first instance to find out whether stearic acid yielded unsaturated acids on oxidation.

At first it was thought that this was so, an acid giving a lead salt soluble in ether being isolated from the products of oxidation. This acid absorbed iodine, but not in amount sufficient to account for one double linkage, and in other ways was different from the unsaturated acids of the oleic series. The investigation of this substance has not been completed but in addition to this acid (or mixture of acids) another new acid was isolated which proved on further investigation to be γ-keto-stearic acid. This showed therefore that besides β oxidation, γ oxidation also takes place. The γ-keto-acid could not be isolated quantitatively so that it is not possible to compare accurately the extent of oxidation at the β and γ carbon atoms. The β-keto-acid, under the

conditions of the oxidations, will decompose practically quantitatively to yield its corresponding ketone and this can be easily estimated. The amount obtained in several experiments indicated that β oxidation of the stearic acid had occurred to the extent of about 6 %. The amount of γ-keto-acid isolated corresponds to about 1 %, but this must be regarded as a minimum figure. In these experiments a large part of the stearic acid is recovered unchanged. It seems probable that other keto-acids are also produced. This certainly happens when the lower fatty acids are oxidised and will be referred to presently, but with the higher acids, their isolation from the large amount of unchanged fatty acid has not been achieved.

In order to see whether other homologues of stearic acid yielded a γ-keto-acid on oxidation, experiments were carried out on similar lines with palmitic and myristic acid. From both these acids γ-keto-acids were obtained and their identity has been confirmed by synthesis[1]. These results with the higher fatty acids suggested the extension of the enquiry to some of the lower members of the series, and the oxidation of hexoic, heptoic and octoic acids has been investigated. The separation of the products of oxidation yielded by these acids proved somewhat troublesome and so far is probably incomplete, but it has been satisfactorily established that each of them, besides undergoing β oxidation, as was shown by Dakin, also undergoes γ and δ oxidation. This has been proved by the isolation of the γ- and δ-keto-acids themselves, or their derivatives, from the products of oxidation and by their identity with specimens obtained by synthesis from substances of known structure. So far as can be determined from the results with these acids, β, γ and δ oxidations take place to about the same extent. Oxidation of fatty acids at the γ carbon atom has been previously observed with α-methylbutyric acid [Raper, 1914] and butyric acid [Cahen and Hurtley, 1917] when hydrogen peroxide was the oxidising agent. In both these instances it seemed likely that the terminal methyl group was oxidised because of its exposed position at the end of the carbon chain, especially as terminally oxidised fatty acids have been found in nature: *e.g.* hydroxypalmitic (juniperic) acid $CH_2OH(CH_2)_{14}COOH$ and hydroxylauric (sabinic) acid $CH_2OH(CH_2)_{10}COOH$ have been isolated from the waxes of certain coniferae [Bougault, 1910]; but the results described in the present paper suggest that oxidation of the γ carbon atom is a general feature when fatty acids are oxidised, and these two instances, therefore, are not exceptional. It is also of interest to note that Bödtker [1912] isolated γ-keto-sebacic acid in small amount when sebacic acid was oxidised with strong nitric acid.

Although hydroxy-acids are almost insoluble in light petroleum and should therefore be separable easily, it was never found possible to detect even traces of these acids; it was therefore concluded that the first step in the oxidation of a saturated acid is probably the production of a series of keto-acids and not hydroxy-acids.

[1] For these syntheses we are greatly indebted to Professor and Mrs Robinson.

The further stages in the oxidation of γ- and δ-keto-fatty acids are at present under investigation; but one significant fact has already been discovered, namely, that both yield succinic acid as one of their oxidation products. This means that the carbon chain in these acids may break between the γ and δ carbon atoms. Four carbon atoms are thus split off as succinic acid.

These results are of interest from the biological point of view. They suggest that in the saturated acids, the β, γ and δ carbon atoms, and possibly also the α, are equally susceptible to oxidation, and that there is no marked difference in polarity between them as regards this property, though there certainly appears to be a marked one when ease of substitution by halogens is the criterion of polarity.

Whether all these forms of oxidation take place in the body we do not yet know. β oxidation certainly does, but there is practically no evidence in favour of α oxidation [Dakin, 1922]. If we accept the view that a substance placed in an environment with a suitably high oxidation potential is oxidised in a certain way which depends upon the structure of the substance and not upon the method by which the oxidation potential is obtained, then results obtained *in vitro* may be provisionally accepted as likely to take place in the body unless modified by some selective agency. That some such agency prohibits α oxidation seems very likely in view of the results of *in vivo* experiments, but the same cannot yet be said of γ and δ oxidation. If either of these processes occurred and were followed by rupture of the fatty acid chain between the γ and δ carbon atoms with the production of succinic acid, then four carbon atoms would be removed at a time instead of the usual two which are postulated by the β oxidation theory of breakdown. Such a process, associated also with β oxidation, would be quite compatible with the well-known results obtained by Embden and his co-workers [1906, 1908] with the surviving liver perfused with blood containing salts of the lower fatty acids. It would also be compatible with the results of Knoop [1904] and Dakin [1909] who showed that the oxidation of phenylvaleric acid in the body yielded benzoic acid. That a γ-keto-acid may lose four carbon atoms at a time on oxidation in the body is suggested very strongly by the results of Knoop and Oeser [1914], who determined the fate in the body of benzyllaevulinic acid. When 15 g. of this acid were administered to a dog, 3·6 g. of phenaceturic acid and 0·8 g. phenyl-α-hydroxybutyric acid were isolated from the urine. No hippuric acid was found. A study of the formula of benzyll-aevulinic acid

$$\underset{\epsilon}{C_6H_5}.\underset{\delta}{CH_2}.\underset{\gamma}{CH_2}.\underset{\beta}{CO}.\underset{\alpha}{CH_2}.CH_2.COOH,$$

shows that β oxidation would yield benzylpyruvic acid,

$$C_6H_5.CH_2.CH_2.CO.COOH,$$

which on reduction would give the phenyl-α-hydroxybutyric acid obtained in this experiment. That the fatty acid side chain did not split between the β and γ carbon atoms and so yield phenylpropionic acid is shown by the

absence of benzoic (or hippuric) acid among the products recovered from the urine, since phenylpropionic acid yields benzoic acid when oxidised in the body. The main product isolated, namely, phenylacetic acid (excreted as phenaceturic acid) seems undoubtedly to have been produced by splitting of the chain between the γ and δ carbon atoms, the δ carbon being oxidised to carboxyl. Presumably the remaining four carbon atoms would be split off as succinic acid which is known to be readily oxidised in the body.

The production of succinic acid by the oxidation of fatty acids in the body would provide a means of explaining how fatty acids might give rise to carbohydrates. Succinic acid is known to be easily converted into fumaric and malic acids both in muscle and liver and the latter acid in phlorhizin diabetes is converted into glucose. Whether this takes place *via* lactic acid is uncertain, but if it does, then a process of oxidation of fatty acids can be visualised which, by yielding in turn succinic acid, malic acid and either lactic acid or glucose, would provide a scheme whereby fat could supply the energy for muscular exercise. There is no experimental evidence which shows that muscle can use fat directly and it appears that it must undergo some preliminary oxidative processes elsewhere in the body in order that it may be prepared for use in the muscle mechanism. The oxidative breakdown of fatty acids must therefore follow some scheme which provides substances which will be utilisable by muscle, *e.g.* lactic acid or carbohydrate. The scheme of β oxidation cannot be said to do this. It does not throw any light on the mechanism whereby fat can be transformed into carbohydrate, as it apparently must be if the current ideas on the working of the muscle mechanism are correct. The very common occurrence in the body of the enzyme succinoxydon, discovered by Batelli and Stern [1911], and its rapid action certainly seem to suggest that there is a greater use for it than the oxidation only of glutamic acid after this has undergone previous oxidative deamination to succinic acid.

Some of these problems and speculations which have necessarily arisen as a result of the experiments described in this paper are being investigated in the hope that they will throw light on some aspects of the metabolism of fatty acids which are at present obscure.

Experimental.

Oxidation of stearic acid. Two samples of pure acid (m.p. 69°, m.w. by titration in neutral alcohol 284, iodine number 0·1), obtained from Messrs Kahlbaum and Poulenc Frères, were oxidised and gave the same result. Stearic acid (10 g.), water (250 cc.), and ammonia (4 cc. 23 %) were heated under reflux in a 3 litre flask to 60° in a water-bath. Hydrogen peroxide (21 cc. 30 %) was then added and the temperature gradually raised to 90° (there is considerable frothing). Heating was continued for 4 hours. The liquid was acidified with phosphoric acid, cooled and the precipitated acids and ketone filtered off. The precipitate was converted into soaps with potash in 50 %

alcohol and the solution extracted five times with light petroleum, thus removing the β oxidation product, methylquindecyl ketone. The alcoholic solution of the soaps was then acidified with dilute sulphuric acid, diluted, cooled and filtered. The resulting acid was recrystallised once from a small quantity of methylated spirit when 6–7 g. of nearly pure stearic acid separated. The acid in the mother liquid was neutralised with soda, poured into water and the lead salts precipitated by adding 20 % lead acetate solution. The ether-soluble lead salts have not been fully dealt with. The lead salts insoluble in ether were converted into acids and the latter crystallised from light petroleum. The first fraction was shown to be a new acid which after three recrystallisations from light petroleum melted at 97°. This acid was also obtained directly by crystallisation of the mother liquor acids from light petroleum without the precipitation of the lead salts. A number of these oxidations were carried out and the high melting fraction accumulated until sufficient was obtained for complete analysis and identification, when it was shown to be γ-ketostearic acid. This acid was synthesised from stearolactone and melts at 97° and on mixture with the acid obtained by oxidation did not depress the melting point. Both acids gave an oxime, M.P. 84°, and the analysis and molecular weight of the acid produced by oxidation corresponded with the theoretical.

(Found C = 72·59, 72·63, H = 11·61, 11·59 %, $C_{18}H_{34}O_3$ requires C = 72·48, H = 11·4 %.)

A trace of another acid (possibly δ-ketostearic acid) was obtained, but not in sufficient quantity for identification. The amount of β and γ oxidation products actually separated and weighed represented about 6 % and 1 % respectively of the stearic acid used. The β figure is probably maximal whereas the γ figure is low, due to the difficulty of separation and to the fact that γ-ketostearic acid is readily further oxidised by peroxide. This latter oxidation is now being investigated. The constitution of γ-ketostearic acid has already been proved by the Beckmann reaction on its oxime [Shukov and Schestakov, 1903].

Oxidation of palmitic acid. Following the above method, an acid melting at 91–92° was isolated. This acid was more soluble in alcohol and less soluble in light petroleum than palmitic acid and corresponded in properties with γ-ketopalmitic acid which was kindly synthesised for us by Mrs Robinson [1925]. The mixed melting point was 91–92°.

Oxidation of myristic acid. The oxidation of this acid was carried out as above but owing to the lower melting point of the acid and the greater solubility in alcohol, the method of separation had to be modified. After separation of the ketone in the usual way, the fatty acids were crystallised from methyl alcohol, the liquid being cooled in ice whereby 60–70 % of unchanged myristic acid was recovered. The acid recovered from the mother liquor was crystallised from 80 % methyl alcohol and yielded a further amount of myristic acid. The acids remaining in the mother liquor were now crystallised

from light petroleum and gave an acid, M.P. 87°, M.W. by titration 244. Calculated for $C_{14}H_{26}O_3$, 242. This acid has been synthesised by Mrs Robinson and from the M.P. and mixed M.P. has been proved to be γ-ketomyristic acid.

Oxidation of n-*hexoic* (*caproic*), n-*heptoic* (*oenanthic*) *and* n-*octoic* (*caprylic*) *acids.* The oxidations were carried out as before, the quantities employed being: acid (10 g.), water (250 cc.), ammonia (20 cc. 23 %), and hydrogen peroxide (50 cc. 30 %). As these acids are liquid at the ordinary temperature the fractional crystallisation method of separation had to be abandoned. In each case, the product of oxidation was extracted five times with light petroleum to remove the *n*-propyl-, *n*-butyl-, *n*-amyl-, methyl ketones formed by β oxidation. The liquid was acidified and again extracted five times with light petroleum. This removed the unchanged acid almost completely together with a trace of keto-acid. The resulting liquid was then extracted in a continuous ether extractor for 5–6 hours and the ethereal solution dried and fractionated. Some acetic acid was obtained in each case and also a main fraction boiling from 170–190° (20 mm.). This was shown to contain the γ- and δ-keto-acids. These were converted into the semicarbazones by adding to the oily fraction a little water, a considerable amount of crystalline sodium acetate and finally semicarbazide hydrochloride. The semicarbazones separated, were filtered off, dried and weighed. From the weight of ketones and of semicarbazones it was calculated that roughly 6 % of the acid had undergone β oxidation and 12 % $\gamma + \delta$ oxidation, and from the fractionation of the semicarbazones it appears probable that the amounts of β, γ and δ oxidation are roughly equal. The melting points (with decomposition) of semicarbazones are known to vary with the rate of heating, but by standardising the latter quite consistent results could be obtained [cf. Vorländer, 1897]. In the case of *hexoic* acid, the keto-acids obtained by oxidation gave a very strong iodoform reaction indicating the presence of δ-ketohexoic acid. The semicarbazones were separated by a long and tedious series of fractional crystallisations from methylated spirit into a least soluble portion, melting at 173°, shown to be δ-ketohexoic acid semicarbazone, a large number of intermediate fractions melting from 144–153° and a most soluble portion, melting at 170°, shown to be γ-ketohexoic acid semicarbazone. The following melting points and mixed melting points show very definitely that these acids were isolated as semicarbazones in practically pure condition:

Ketohexoic acid semicarbazone	M.P.
γ (synth.), γ (oxidn.)	170°
γ (synth.) + γ (oxidn.)	170
δ (synth.), δ (oxidn.)	173
δ (synth.) + δ (oxidn.)	173
δ (synth.) + γ (synth.)	145–148
δ (synth) + γ (oxidn.)	145–148
γ (synth.) + δ (oxidn.)	145–148
γ (oxidn.) + δ (oxidn.)	145–148

Dumas nitrogen estimations on the semicarbazones of the oxidation γ- and δ-ketohexoic acids thus separated, gave the following results:

(*a*) γ: found N = 22·3 %; $C_7H_{13}O_3N_3$ requires N = 22·4 %.

(*b*) δ: found N = 22·2 %; $C_7H_{13}O_3N_3$ requires N = 22·4 %.

The oximes of the synthetic γ-ketohexoic and δ-ketohexoic acids and of the oxidation mixture of these two acids were made by dissolving hydroxylamine hydrochloride (5 g.) in a small amount of water, adding the keto-acid (6 g.) and, after cooling, mixing with potash (7 g. in a little water) and allowing to stand overnight. The liquid was then acidified, saturated with ammonium sulphate and extracted in a constant ether extractor for 2 or 3 hours. The ethereal solution was dried over calcium chloride and the ether evaporated off. In the case of δ-ketohexoic acid, the oxime readily solidified and was recrystallised from benzene; M.P. 104°. In the case of γ-ketohexoic acid a syrup was obtained which, after standing 3 days over sulphuric acid *in vacuo*, on scratching with a glass rod gradually set to a solid. This was pressed on a porous plate and a little oily substance thus removed. The oxime was dissolved in dry ether, the solution filtered and then precipitated with light petroleum. After several purifications it melted at 67°. In the case of the keto-acids from the oxidation products the oxime was a thick syrup. This was dissolved in benzene and the oximes precipitated with light petroleum as an oil which, after inoculating with a very small crystal of δ-ketoxime, was allowed to stand 3 days *in vacuo* over sulphuric acid. Partial solidification took place and the mass was placed on a clean porous plate. The syrup was absorbed and a white solid residue obtained which crystallised readily from benzene, melted at 104°, and, mixed with synthetic δ-ketoxime, showed no depression of melting point. The absorbed syrup was recovered by extraction of the porous plate with benzene and the oxime precipitated with light petroleum and the process repeated. A little more δ-ketoxime was obtained and the syrup recovered after this operation was finally caused partially to solidify after inoculating with a small crystal of synthetic γ-ketoxime and allowing to stand *in vacuo* for several days. This fraction after purification was shown by mixed melting point to be the γ-ketoxime.

Analysis.

(*a*) γ: found N = 9·4 %; $C_6H_{11}O_3N$ requires N = 9·7 %.

(*b*) δ: found N = 9·5 %; $C_6H_{11}O_3N$ requires N = 9·7 %.

In the case of *heptoic acid*, the semicarbazones of the oxidation keto-acids were separated as above and gave a less soluble first portion, M.P. 194°, identical with the synthetic δ-ketoheptoic acid semicarbazone and a much more soluble one, M.P. 152°, identical with γ-ketoheptoic semicarbazone. The mixed melting points of oxidation and synthetic semicarbazones were taken and the Dumas

nitrogen analysis of the oxidation acid semicarbazones gave the following results:

(*a*) γ: found N = 20·8 %; $C_8H_{15}O_3N_3$ requires N = 20·9 %.

(*b*) δ: found N = 21·1 %; $C_8H_{15}O_3N_3$ requires N = 20·9 %.

The ketoheptoic acids obtained by oxidation gave a slight iodoform reaction suggesting that a little ϵ oxidation had taken place. It was not found possible however to isolate with certainty a pure sample of the ϵ-semicarbazone (M.P. 144°) from the semicarbazone mixture.

In the case of *octoic acid*, similarly, the semicarbazone mass was by a tedious series of fractionations from alcohol separated into a less soluble first fraction of δ-ketooctoic acid semicarbazone, M.P. 187°, and a much more soluble fraction, γ-ketooctoic acid semicarbazone, M.P. 153°, neither of which depressed the M.P. of the corresponding synthetic semicarbazone. The following Dumas nitrogen determinations were carried out on the oxidation semicarbazones:

(*a*) γ: found N = 19·8 %; $C_9H_{17}O_3N_3$ requires N = 19·5 %.

(*b*) δ: found N = 19·7 %; $C_9H_{17}O_3N_3$ requires N = 19·5 %.

Synthesis of keto-acids by means of zinc alkyl iodides.

The method adopted was similar to that of Blaise and Kohler [1910]:

$$RZnI + ClCO(CH_2)_nCOOEt = RCO(CH_2)_nCOOEt + ZnICl.$$

(i) *Preparation of the zinc alkyl iodides.*

Pure zinc filings (90 %) and pure copper filings (10 % of total weight) were heated together in a dry flask in the yellow blowpipe flame until the mass fell to a black powder. The alkyl iodide (1 mol.), ethyl acetate ($\frac{1}{3}$ mol., dried for several days over calcium chloride, distilled and first and last fractions neglected), dry redistilled toluene (double the weight of ethyl acetate) and Zn – Cu couple (double the amount required by the iodide) were heated in a dry flask under reflux at 100–110° for 1½ hours. A second equal amount of toluene was added and, after shaking, the flask was cooled. The white fuming liquid was poured into a dry flask and the couple washed with a little dry toluene. In this way were prepared zinc ethyl-, *n*-propyl-, *n*-butyl-, iodides.

(ii) *Preparation of half ester half acid chlorides of*

(*a*) *Succinic acid.* Succinic anhydride (1 mol.) was boiled with methyl alcohol (2 mols.) for 45 minutes. The excess of alcohol was distilled off and the mass after solidifying was crystallised from a large quantity of carbon disulphide. The monomethyl ester (M.P. 58°) thus obtained was boiled with three times its weight of redistilled thionyl chloride, the excess of thionyl chloride distilled off *in vacuo* and the methyl ester chloride (B.P. 93°/18 mm.) was collected.

(*b*) *Glutaric acid.* Glutaric acid was prepared by a method, the details of which were kindly provided by Professor C. K. Ingold. Cyclohexanol

(500 g.) was added, drop by drop, with occasional shaking, to 2700 g. conc. nitric acid in a 5 l. flask provided with an efficient reflux condenser and heated in a boiling water-bath. After the reaction was completed (4–5 hours) the liquid was poured into a basin and allowed to stand overnight. The crystals of adipic acid were filtered off on a glass wool pad in a large Buchner funnel and thoroughly dried in the steam oven, powdered and heated in the oven for 48 hours completely to drive off the nitric acid. A little more was obtained from the mother liquors making the total yield of adipic acid 558 g. This experiment was repeated. Dry adipic acid (300 g.) and baryta (50 g.) were heated in a 1·5 l. distilling flask in an air-bath at 290°. The water coming over at first was neglected and the cyclopentanone collected as long as any liquid distilled (3–4 hours). This was shaken with ether, when a little water separated, and the ethereal solution was dried over calcium chloride and distilled, using a long fractionating column. The cyclopentanone collected at 129–133° weighed 118 g. This experiment was repeated. Cyclopentanone (200 g.) was dropped (as in the oxidation of cyclohexanol) into 2000 cc. of dilute nitric acid (sp.g. 1·2). The resulting liquid was evaporated to dryness, thoroughly dried and freed from nitric acid. The powder obtained was placed in a large Soxhlet thimble and suspended in a flask over boiling benzene, the condensed benzene dripping into the thimble. Almost pure glutaric acid, M.P. 95–96°, separated from the benzene solution on cooling. The extraction was continued until only succinic acid remained in the thimble. 1 kg. cyclohexanol gave 390 g. glutaric acid. Glutaric anhydride was prepared by heating dry glutaric acid with five times its weight of thionyl chloride, evaporating off the excess of the latter *in vacuo* and collecting the fraction boiling at 145–165°/20 mm. This fraction readily solidified and was crystallised from boiling ether when it melted at 55°. The anhydride was boiled with double the theoretical amount of methyl alcohol, the excess of alcohol was distilled off and the monomethyl ester collected at 153°/20 mm. This was boiled with three times its weight of redistilled thionyl chloride, excess of the latter was removed *in vacuo* and the methyl ester chloride collected at 110°/20 mm.

(iii) *Preparation of the keto-acids.*

The methyl ester acid chloride dissolved in its own volume of dry toluene was added drop by drop to the zinc alkyl iodide solution, the latter being about 25 % in excess for the acid chloride used. During the whole operation the reaction flask was kept in ice and shaken constantly. After standing, water was added, a little at a time at first, shaking vigorously. A little precipitated zinc hydroxide was dissolved in dilute sulphuric acid. The toluene solution was separated and washed with dilute ammonia, then with dilute sulphuric acid and finally with dilute bicarbonate and thiosulphate. The solution was dried over anhydrous sodium sulphate and fractionated *in vacuo*. The methyl esters of the keto-acids were then hydrolysed by shaking with strong potash, the product acidified and extracted with ether. (In the case of

γ-ketohexoic acid continuous ether extraction was necessary.) The ethereal solution was dried and fractionated *in vacuo*. In this way were prepared:

(*a*) *γ-ketohexoic acid*; using zinc ethyl iodide and the half-ester half-acid chloride of succinic acid. The acid was collected from 150–180°/20 mm. and set to a hard mass on standing *in vacuo*. This was dissolved in benzene, filtered from a trace of succinic acid and the benzene evaporated to a small volume, when the acid crystallised. Purification by precipitation from benzene solution by light petroleum was also tried. γ-Ketohexoic acid melts at 32°, boils at 180°/20 mm. and gives a semicarbazone melting with decomposition at 170°, which appears to be identical with that obtained by Campbell and Thorpe [1910]. The oxime melts at 68°. The methyl ester boils at 110°/20 mm.
Analysis.

Semicarbazone: found N = 22·35 %; $C_7H_{13}O_3N_3$ requires N = 22·4 %.
Oxime: found N = 9·9 %; $C_6H_{11}O_3N$ requires N = 9·7 %.

(*b*) *γ-ketoheptoic acid*; using zinc propyl iodide and the half-ester half-acid chloride of succinic acid. The acid melts at 48° and gives a semicarbazone, M.P. (decomp.) 152°. The methyl ester boils at 125°/20 mm.
Analysis.

Semicarbazone: found N = 21·1 %; $C_8H_{15}O_3N_3$ requires N = 20·9 %.

(*c*) *γ-ketooctoic acid*; using zinc butyl iodide and the half-ester half-acid chloride of succinic acid. The acid melts at 53° and gives a semicarbazone, M.P. (decomp.) 153°. The methyl ester boils at 111°/15 mm.
Analysis.

Semicarbazone: found N = 19·8 %; $C_9H_{17}O_3N_3$ requires N = 19·5 %.

(*d*) *δ-ketoheptoic acid*; using zinc ethyl iodide and the half-ester half-acid chloride of glutaric acid. The acid melts at 50° and gives an oxime M.P. 118° and a semicarbazone, M.P. (decomp.) 194°. The methyl ester boils at 130°/20 mm.
Analysis.

Semicarbazone: found N = 21·2 %; $C_8H_{15}O_3N_3$ requires N = 20·9 %.

(*e*) *δ-ketooctoic acid*; using zinc propyl iodide and the half-ester half-acid chloride of glutaric acid. The acid gives a semicarbazone, M.P. (decomp.) 187°. The methyl ester boils at 120°/15 mm.
Analysis.

Semicarbazone: found N = 19·7 %; $C_9H_{17}O_3N_3$ requires N = 19·5 %.

Synthesis of δ-ketohexoic acid.

The method adopted was essentially that of Bentley and Perkin [1896]. Sodium (3·4 g.) was dissolved in alcohol (45 g.) and to the well-cooled solution acetoacetic ester (19 g.) and then β-iodopropionic ester (33 g.) were added. After standing for half an hour the whole was heated on a water-bath for

2 hours, water added and the oil extracted with ether. The ethereal solution was washed with a little water containing SO_2, dried over $CaCl_2$, evaporated and the oil fractionated *in vacuo*. Acetylglutaric ester was collected at 190–200°/50 mm. and boiled under reflux with five times its volume of dilute (1 : 2) HCl for 5 hours. The liquid was saturated with ammonium sulphate and extracted six times with ether. The ethereal solution was dried over $CaCl_2$ and fractionated, the keto-acid being collected at 195–200°/65 mm. The acid is an oil which forms a crystalline monohydrate, M.P. 35°, and very readily gives the iodoform reaction. The semicarbazone melts at 173° and the oxime at 103°.

Analysis.

Semicarbazone: found N = 22·6 %; $C_7H_{13}O_3N_3$ requires N = 22·4 %.
Oxime: found N = 9·6 %; $C_6H_{11}O_3N$ requires N = 9·7 %.

Synthesis of γ-ketostearic acid. Stearolactone, M.P. 52°, was prepared by the action of sulphuric acid on oleic acid [Schukov and Schestakov, 1908] and oxidised to γ-ketostearic acid by means of chromic acid in acetic acid solution [Clutterbuck, 1924].

The acid melts at 97° and was converted into its oxime as follows: γ-ketostearic acid (1 mol.), hydroxylamine hydrochloride (1 mol.) and caustic soda (3 mols.) in alcoholic solution were boiled for 2 hours on the water-bath. The alcohol was distilled off, the residue dissolved in water and, after cooling, made just acid. The precipitated acids, after extraction with ether and removal of the latter, were boiled with light petroleum in which the oxime was more soluble than the unchanged keto-acid. The oxime when pure melted at 85°. The reaction was studied under varying conditions but the yield was never theoretical and consequently oxime formation could not be used for the quantitative estimation of γ-ketostearic acid in the oxidation mixture.

Analysis. Found N = 4·29 %; $C_{18}H_{35}O_3N$ requires N = 4·49 %.

γ-Ketostearic acid phenylhydrazone was prepared by dissolving the keto-acid in warm glacial acetic acid and adding a few drops of phenylhydrazine. The hydrazone crystallised out on standing and was recrystallised from light petroleum. It melts with total decomposition at 80° and therefore great care was required in its purification.

Analysis. Found N = 7·0 %; $C_{24}H_{40}O_2N_2$ requires N = 7·2 %.

Synthesis of γ-ketopalmitic and γ-ketomyristic acids. These were kindly prepared by Mrs Robinson [1925]. The former melts at 91–92° and gives an oxime, M.P. 54°. The latter melts at 87°.

SUMMARY.

(1) The ammonium salts of normal saturated fatty acids when oxidised with hydrogen peroxide yield products which show that oxidation occurs at the γ and δ carbon atoms as well as the β carbon atom (and possibly also the α).

(2) From myristic, palmitic and stearic acids, the corresponding γ-keto-acids have been isolated as products of oxidation.

(3) From *n*-hexoic, *n*-heptoic and *n*-octoic acids the corresponding γ- and δ-keto-acids have been isolated as semicarbazones and in some cases also as oximes. Several of these acids had not been previously prepared and have been synthesised for reference.

(4) The possibility of the occurrence of γ and δ oxidation in the body is discussed and it is pointed out that if further investigation proves that it does occur, some light may be thrown on the processes by which fat may be utilised to supply energy for the contraction process of muscle.

(5) The first step in the oxidation of normal saturated fatty acids appears to be the formation of a series of keto-acids and not of hydroxy-acids. The latter, although almost insoluble in light petroleum, could not be detected. This would support the view that the hydroxy-acids obtained in experiments *in vivo* result from reduction of keto-acids rather than from direct oxidation of saturated acids.

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LVIII. STUDIES ON THE PHYSIOLOGY OF PLAIN MUSCLE.

III. COMPARISON OF THE REDUCING PROPERTIES OF PLAIN AND STRIATED MUSCLE[1].

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The purpose of the present experiments was to study the reducing properties of plain and striated muscle in order to ascertain whether there were any pronounced differences in this respect which would be of value in the consideration of their comparative physiology. In making the comparison, the samples of plain and striated muscle have in each case been taken from the same animals, sometimes the frog, more often a mammal, and have been subjected to identical treatment. The actual striated muscle chosen for comparison has varied; in cats the muscle of the thigh, in frogs the sartorius or gastrocnemius, in the bullock, portions of perineal muscle, etc. In view of the greater variability of plain muscle, as regards its physiology, various sources have been used, but no conspicuous differences between them were seen. The tissues used were small intestine, uterus, stomach, and the retractor penis. The reductions studied were those of methylene blue, and of *m*-dinitrobenzene, the former by the well-known technique introduced by Thunberg [1920], the latter as carried out by Lipschitz [1920]. When possible the experiments were carried out on fresh tissues; when material had to be kept it was immersed in 2 % boric acid and kept in the refrigerator; previous to use such preserved muscle was well washed with several changes of distilled water in a shaking machine; such material served very well for the study of the reductions of washed tissue.

It may be said at the outset that the qualitative differences which were found between the two varieties of muscle were negligible, the only important differences being quantitative.

The Reduction of Methylene Blue.

The reduction of methylene blue *in vacuo* at 35° is usually effected by plain muscle in the fresh state with a velocity only about one-third to one-fifth

[1] Protocols of experiments have been abbreviated or omitted in order to save space, as the technique employed is fully described in the papers quoted.

of that brought about by striated muscle under identical conditions. The following table shows the result of a typical experiment:

Table I. *Reduction of Methylene Blue by Fresh Muscle.* (*Cat. Intestine and thigh muscle.*)

Mg. m. bl.	0·02	0·02	0·04	0·04	0·06	0·06	0·08	0·08
Plain mus. (g.)	0·2	—	0·2	—	0·2	—	0·2	—
Str. mus. (g.)	—	0·2	—	0·2	—	0·2	—	0·2
Time for reduction (mins.)	15	4	11	12	40	—	40	17
Mg. m. bl.	0·04	0·04	0·04	0·04	0·04	0·04	0·04	0·04
Plain m. (g.)	0·1	—	0·2	—	0·3	—	0·4	—
Str. mus. (g.)	—	0·1	—	0·2	—	0·3	—	0·4
Time for reduction (mins.)	>43	21	28	5	27	5	12	1

It will be seen that the plain muscle is much inferior in its reducing power to striated muscle, both when compared with varying amounts of the dye, and also when the amount of tissue is changed, the concentration of dye being kept constant. This result is not surprising in view of the relatively small oxygen usages of plain muscle reported by Evans [1923]. Similar results were obtained with frog tissues; thus in one experiment, using 0·2 g. tissue and 0·4 mg. methylene blue, the times for reduction were 40 minutes for plain muscle, 7·5 minutes for heart muscle, and 5·5–8·0 minutes for striated muscle.

When the finely minced plain muscle is well washed with several changes of water, its reducing power is almost completely lost; this happens also with the striated muscle. The reduction can, however, be restored again to a considerable extent by addition of potassium succinate as also holds good for striated muscle. One sample of plain muscle (0·2 g.) for instance in the fresh state reduced 0·04 mg. methylene blue in 28 minutes; after washing well with water the same tissue (0·2 g.) showed no sign of reduction of 0·02 mg. methylene blue after 1 hour; when 0·2 cc. of 0·1 molar potassium succinate was added, the reduction under otherwise similar conditions was almost complete in 50 minutes.

Optimal Reaction.

When buffered with phosphate solutions, the optimal rate of reduction was obtained with striated muscle at a p_H above 10·5, whilst with plain muscle the optimum was about p_H 9·0–10·0. With borate buffers the optimal p_H was about 8·0 for plain, and about 8·8 for striated muscle. In both instances washed tissue in presence of succinate was used for these tests. The following is the result of the experiments: in each case 0·2 g. of the washed tissue, 0·1 cc. 1 : 5000 methylene blue, 0·1 cc. of $M/10$ potassium succinate, and 0·9 cc. of $M/10$ potassium phosphate buffers were taken. The reduction times were:

p_H	For striated muscle	For plain muscle
3·0–6·7	>60′	>72′
7·2	27′	61′
7·7	28′	37′
9·0	24′	26′
10·5	15′	36′

Alteration in the amount of buffer solution made no appreciable difference to the result, *e.g.* the reduction times were the same with 1 cc. of buffer solution as with 0·2 cc. buffer solution and 0·8 cc. water.

Effect of Temperature.

The optimal temperature, at p_H 9·0 is, according to my experiments, about 50° for both varieties of muscle. Thus in a mixture containing 0·2 g. washed muscle, 0·1 cc. of 1 : 5000 methylene blue, 0·8 cc. of *M*/10 phosphate buffer, and 0·1 cc. of *M*/10 potassium succinate, the reduction times (brought to the same scale for purposes of comparison where different samples were used) were as follows: at 0°, the reduction was scarcely apparent, even after 18 hours; at 20° it was partially complete in 4 hours, at 30° complete in 4 hours, at 40° in 50 minutes, at 50° in 23 minutes, and at 60° in 32 minutes. Experiments were, however, usually carried out at 38°.

Effect of Salts and of KCN.

The reduction of methylene blue by washed plain muscle, in presence of succinate, is accelerated by both sodium and potassium chlorides, precisely as with striated muscle. The effect of cyanide is also identical, that is to say, the reduction by the washed tissue (0·2 g.) of methylene blue (0·2 cc. of 1 : 5000) in presence of succinate (0·2 cc. of *M*/10 potassium succinate) is not inhibited, but slightly, though definitely, accelerated by the presence of potassium cyanide in concentrations up to 0·2 cc. of *M*/100 KCN in 1 cc. of reaction mixture. The reduction was carried out in presence of 0·4 cc. of *M*/10 K_2HPO_4, and the times for complete reduction were, in one experiment, without cyanide 2 hours; with 0·1 cc. *M*/1000 cyanide, 1 hour 24 minutes; with 0·2 cc. *M*/1000, 1 hour 13 minutes, with 0·05 cc. *M*/100, 1 hour 19 minutes; with 0·1 cc. 1 hour 11 minutes, and with 0·2 cc., 1 hour 15 minutes. Other experiments gave similar results, there being in all cases definite acceleration of the reduction after addition of KCN. The acceleration was possibly due to slight reduction of the H-ion concentration of the mixture, but was not further investigated, the chief point being that there was no inhibition of the reduction. In this respect also there is a close resemblance to the behaviour of striated muscle, as studied by Thunberg [1917].

Restoration of Reducing Power by Addition of Organic Acids.

When plain muscle is washed in the manner described by Thunberg [1920], its reduction power is almost completely lost. On addition of potassium succinate the reducing power is partially restored; but no matter how much succinate is added to it, the reducing power does not approximate to that of the fresh tissue, and still less to that of fresh striated muscle.

It appeared of interest, in connection with the results obtained by Thunberg, to find whether the substances which were capable of restoring the power

of reduction to plain muscle inactivated by washing were the same as those which acted in that way with washed striated muscle. In addition to succinate, I tried lactate, citrate, acetate and formate. The experiments were carried out in every respect according to the Thunberg technique, and need not be described in any detail; in each instance a parallel experiment was carried out with washed striated muscle to serve as a control. The amounts of materials placed in the evacuated tubes were as follows: muscle 0·2 g. $M/10$ potassium lactate (or other organic salt) solution and water together to make up 0·4 cc.; $M/10$ K_2HPO_4 solution 0·5 cc.; methylene blue (1 : 5000) 0·1 cc. Temp. 37°.

The results, at the reaction given by this mixture (about p_H 9) may be summarised as follows:

Plain muscle		Striated muscle	
Reduction time		Reduction time	
No lactate + +in 4 h. 19 m.	Lactate (0·4 cc.) Complete in 4 h. 9 m.	No lactate + +in 4 h. 7 m.	Lactate (0·2 cc.) Complete in 4 h. 2 m.
No formate 0 in 2 h.	Formate (0·1–0·4 cc.) 0 in 2 h.	No formate 0 in 2 h.	Formate (0·1–0·4 cc.) 0 in 2 h.
No acetate +in 3 h.	Acetate (0·1–0·4 cc.) +in 3 h.	No acetate +in 3 h.	Acetate (0·1–0·4 cc.) +in 3 h.
No citrate + +in 3 h. 27 m.	Citrate (0·4 cc.) +in 3 h. 23 m.	No citrate + + +in 3 h. 22 m.	Citrate (0·5 cc.) + + +in 3 h. 16 m.

These results show that there was a slight acceleration of reduction by lactate, but, at this hydrogen ion concentration no effect produced by acetate, formate or citrate. The effect of altering the H-ion concentration was, however, quite considerable with citrate, which, as shown by Fleisch [1924], is oxidised by washed muscle and methylene blue much more rapidly at a p_H between 5·0 and 6·5 than at 7·6. I have similarly found that whilst at p_H 9·0 and 7·5 citrate was not oxidised, there was pronounced decolorisation of the methylene blue at p_H 6·3 and 5·5, *e.g.* in experiments with quantities as above, and using washed plain muscle:

At p_H 5·5 without citrate; only slight decolorisation in 3 hours 38 minutes.

At p_H 5·5 with 0·1 cc. citrate; decolorisation almost complete in $3\frac{1}{2}$ hours.

At p_H 6·3 without citrate; slight decolorisation in 3 hours 37 minutes.

At p_H 6·3 with 0·3 cc. citrate; almost completely decolorised in $3\frac{1}{2}$ hours.

At p_H 7·5 without citrate, or with 0·6 cc. citrate; decolorisation nearly complete in $3\frac{1}{2}$ hours.

With lactates it was found that the acceleration of decolorisation was most rapid at p_H 8·0–9·2.

There does not seem to be any noteworthy difference between the properties of striated and those of plain muscle in these respects. I could not confirm the oxidation of acetates and formates by either tissue, though a definite action by striated muscle was found by Thunberg [1920].

Reduction of *m*-Dinitrobenzene.

A comparison was also made between the reducing power of plain and striated muscles and of other tissues, on *m*-dinitrobenzene. W. Lipschitz [1920, 1921] and his collaborators have shown that this substance is reduced by many tissues to the soluble, yellow *m*-nitrophenylhydroxylamine, the amount of which may be estimated colorimetrically. This reduction, unlike that of methylene blue, is not reversible by atmospheric oxygen, though it takes place more readily in the absence of oxygen, and in most ways gives results which are similar to those given by methylene blue reduction.

It was found, using a method similar to that of Lipschitz, that plain muscle reduced the *m*-dinitrobenzene with much greater speed than did striated muscle. Comparison of various tissues showed that with cat's tissues the amounts of reduction obtained in equal lengths of time were in descending order: plain muscle of small intestine; heart muscle; uterus muscle; striated muscle. With frog's tissues liver and testis reduced more rapidly than muscular tissue, plain and striated muscle showing little difference in this experiment. In all the experiments with mammalian tissues however it was found that the plain muscle showed upwards of twice as much reduction in a given time as was shown by striated muscle. In all these experiments the amounts of substances used were, muscle 0·5 g., total fluids 3·0 cc. and *m*-dinitrobenzene, in finely powdered form, 0·05 g. It was found that the reduction with striated muscle soon reached a standstill, whereas that given by the plain muscle was able to proceed for a longer time, as well as at a more rapid rate; it seems highly probable that this remarkable difference between the two tissues, which behave in the opposite way towards methylene blue reduction, may be due to the fact that the reduction product is for some reason more toxic to striated muscle than to plain muscle, and so brings the reduction reaction to an end as soon as it has accumulated in sufficient amount. It was found, in agreement with Lipschitz, that the reduction took place in all instances much more rapidly *in vacuo* than when the contents of the tube were exposed freely to the air. On thorough washing with water, the reducing power was completely lost, but was partially restored by addition of succinates, just as with methylene blue reduction. The reduction was not inhibited by reasonably small amounts of cyanide, such as *M*/400 KCN, or less.

This fact is not in agreement with the observations of Lipschitz and Gottschalk [1921] who found considerable inhibition at low concentrations of cyanide with frog's striated muscle.

The Reducing Power of Aqueous Extracts.

Aqueous extracts of fresh plain and striated muscle were made by treating the minced tissue with twice its weight of water, to which some boric acid was added when it was to be kept at room temperature for many hours, or on ice for many days. The extraction was carried out at 0° as a rule, but in some instances at room temperature or at 60–70°. The filtered extracts ob-

tained differed in appearance, that from plain muscle being colourless but opalescent, while that from striated muscle was red, owing to the presence of haemoglobin, and clear. In some of the experiments filtration was carried out through a Berkfeld filter, but the results were the same as when the filtration was through paper alone. The filtered extracts showed definite reducing power towards methylene blue *in vacuo*, but the plain muscle extract had much the more powerful effect in this respect. Thus in one experiment 0·2 g. of fresh plain muscle reduced 0·02 mg. of methylene blue in 1 cc. water in 35 minutes; the same amount of striated muscle brought about the reduction in 8 minutes. An extract of samples of each tissue was made at 60° with twice its weight of water, and filtered after 1 hour. To 0·9 cc. of each filtrate 0·1 cc. of methylene blue (0·02 mg.) was added; reduction occurred *in vacuo* in 21 minutes with the plain muscle extract, whilst the striated muscle extract showed no sign of reduction after 36 minutes. On adding to 0·9 cc. of each extract 0·2 g. of washed muscle from which the extracts had been made, and 0·1 cc. of the methylene blue solution, there was reduction in 15 minutes with the plain muscle extract plus washed plain muscle, and in 20 minutes incomplete reduction with the striated muscle extract to which the washed striated tissue had been added. It was found that plain muscle extract re-activated washed striated muscle much more than striated muscle extract. Parallel with this greater reducing and re-activating power of the plain muscle extract is a much more intense nitroprusside reaction for glutathione [Hopkins, 1921], and the fresh tissues themselves also show the same difference in this respect when tested directly. On boiling, or on being allowed to stand in air for 2 or 3 days the extract loses its reducing power, and no longer shows the nitroprusside reaction; it is however still able to some extent to restore the reducing power to washed tissue. When the boiled extract is treated with a little fresh tissue ($\frac{1}{10}$th its weight), and again filtered off after half an hour, it is found that the reducing power and the nitroprusside reaction have been restored to the extract. It is evident from a consideration of these results that whilst the fresh extract of plain muscle contains a relatively large amount of gluthathione, this is lost by oxidation on standing, but can be converted to the reduced form again by treatment with either fresh or washed tissue [Hopkins, 1921]. It was also found that the reduction of washed tissue was accelerated to a greater extent when plain muscle extract and potassium succinate were added than when either of these was added alone; in fact in one experiment the reduction of methylene blue by washed muscle and fresh extract was no quicker than that by the extract alone.

Apparently some other factor is concerned in addition to glutathione, because it was found that when the extract was heated to 65° for 30 minutes in slightly acid solution, and then neutralised, much of its reducing power had been lost, although the nitroprusside reaction remained as intense as before. This was possibly due to destruction of some soluble catalyser or hydrogen acceptor.

Conclusions.

1. Plain muscle reduces methylene blue more slowly, but *m*-dinitrobenzene more quickly than striated muscle. Neither reduction is inhibited by cyanide.

2. Both types of reduction are almost abolished by washing, but are partly restored by addition of succinate.

3. The optimal p_H for plain muscle reduction of methylene blue is about 9·0.

4. The differences between the reducing action of plain and striated muscle appear to be quantitative rather than qualitative. It seems unlikely that there is any essential difference between them as regards the nature of their intermediary metabolic products.

5. Plain muscle and its extracts are richer in glutathione than striated muscle or its extracts, and show properties in accordance with this.

I hope to continue these experiments in the near future on my return to Japan.

My thanks are due to Professor C. Lovatt Evans for allowing me the facilities of his Department during this investigation.

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LIX. ANIMAL QUINOIDINE.

PRELIMINARY PAPER.

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It is a well-known fact that the nails, skin, teeth and bones fluoresce blue when exposed to a source of ultra-violet radiation. This is best seen by interposing between the source of radiation and the irradiated object a sheet of material which excludes the visible rays. While examining the properties of some of the yeast concentrates obtained in the course of another research, it was observed that these also showed a blue fluorescence. Specimens of normal urine are also known to behave similarly. The coincidence of these facts led to this work. We have found the following literature upon the subject of blue fluorescence in ultra-violet light in the animal organism.

Historical.

Brucke [1845] stated that the lens of the human eye absorbed blue rays. Helmholtz [1855] found the human retina to be feebly fluorescent. Regnauld [1858] using sunlight described a blue fluorescence in the ultra-violet in the lens of the sheep, dog, cat and rabbit, the central part of the lens retaining this property upon keeping. This did not hold for aquatic vertebrates or mollusca. In the case of the human eye, Setchenow [1859] found that the lens fluoresced strongly like quinine, the blue tone predominating. The cornea on the contrary was only feebly fluorescent and the aqueous humour did not fluoresce at all. The most interesting paper is one by Bence Jones [1866]. In a lecture to the Royal Institution, he described the presence of a substance in animal tissues to which he gave the name of "animal quinoidine." In the course of a research upon the rate at which quinine was distributed to the tissues after administration, he found that all animal tissues and organs contained a substance which fluoresced blue when excited by ultra-violet rays. Its behaviour resembled that of quinine in that the fluorescence was diminished by addition of salt or strong acid, being resistant to treatment with permanganate in acid solution. By comparison with the fluorescence of known concentrations of quinine salts he estimated the amount of this substance in various tissues.

Other work upon the nature of blue fluorescent substances seems to be scanty. Schliess and Lowenfeld [1863] described the blue fluorescence of urine in sunlight. Jaffé [1869] mentioned the blue fluorescence of urine, but the paper in question deals mainly with the properties of urobilin and the green fluorescence of bile salts. In modern times Schanz [1921] investigated the fluorescence of the lens and Langecker [1922] stated that most urines show a blue fluorescence and some a yellow. Familiar urinary constituents such as urochrome, uroerythrin, urobilin, and the porphyrins were not considered to be responsible for the blue fluorescence. In the case of the porphyrins, he prepared a series of salts of haematoporphyrin by the Milroy technique most of which gave a yellow or red fluorescence, the only exceptions to this being the copper salt which fluoresced violet in amyl alcohol, and the lead salt which fluoresced green.

Experimental.

The present paper gives an account of certain observations which we have made upon the nature and distribution of substances which fluoresce blue in ultra-violet light (Å. 3000–4000). Our observations are qualitative only so far as this paper is concerned, and may be grouped for convenience under the following headings:

A. Substances of known constitution, and certain commercial preparations.

B. Animal products: (1) (*a*) Guano, (*b*) Urine, (*c*) Bile.
(2) Other animal extracts.
(3) (*a*) Gelatin, (*b*) Skin, (*c*) Lens.

C. Plant products: (1) Grass.
(2) Oils.
(3) Other products.

Throughout we have employed as a source of ultra-violet radiation a mercury vapour quartz lamp made by the Hewittic Company, and have used as a screen for the exclusion of the visible rays a plate of glass supplied by Messrs Chance.

According to the assertion of the makers, this screen lets through light of wave length Å. 3100–3900, the maximum being at a wave length of 3650, at which there is a transmission of some 50 %. Solutions have been examined for fluorescence in thin walled test tubes 13 × 75 mm., having a thickness of glass of some 0·5 mm. Controls in quartz tubes have not shown different behaviour as regards the colour of the fluorescence.

A. *Substances of known constitution, and certain commercial preparations.*

Table I (*a*) is a list of substances of known constitution which have shown no fluorescence in solution. In the case of the amino acids care was taken to examine the behaviour in alkaline and acid as well as in neutral solution. Alcohol was added to obtain solution where necessary.

Table I (*b*) shows certain other substances found to fluoresce a faint blue.

Table I (*a*). *Substances showing no blue fluorescence in solution.*

Tryptophan, cystine, leucine, histidine, tyrosine, glycine.

Allantoin, caffeine, hypoxanthine, uric acid, creatine, creatinine, xanthine, alloxan, parabanic acid, guanine, urea, guanidine, hippuric acid.

Cholesterol, cholesterol acetate, cholic acid, cholalic acid, laevulose, arabinose, cane sugar (purified by alcohol treatment), mannitol, sodium propionate, sodium maleate, salol, salicin, amygdalin, calcium lactate, starch, crotonic acid, tripalmitin, sodium glycerophosphate, turpentine, histamine, triphenylguanidine, choline, ox blood plasma and serum[1], egg albumin.

Table I (*b*). *Substances showing blue fluorescence.*

Guanine (commercial specimen; due to impurity), yeast nucleic acid (comm. specimen), lecithin (comm. specimen; very faint), crude lanolin, sodium glycocholate and taurocholate (laboratory specimens, faint blue): in the case of the glycocholate, it was found that the fluorescence could be removed by shaking with chloroform.

Gelatin, Nelson's "gold label" (commercial).

Gelatin from ossein
Gelatin from Coignet "gold label" } flocculated in an electric field.
Gelatin from horn

(In the flocculated gelatin, the fluorescence was much less decided than in the commercial gelatin.)

B. *Animal products.*

1. (*a*) *Guano.*

We were led to investigate guano extracts from the observation that a commercial specimen of guanine fluoresced a blue colour. Fluorescent extracts can be obtained from guano in several ways of which the following two are typical examples:

Method 1. Guano is extracted with warm 50 % alcohol containing 1 % HCl. From the cooled and filtered extract, the addition of either ether or chloroform extracts the bulk of the blue fluorescent substance, some however always remains behind in the aqueous phase.

Method 2. Guano is extracted with warm acetone. From the cooled and filtered extract practically all the blue fluorescent matter passes into the ethereal layer, upon addition of ether and water, which, upon evaporation, yields a reddish brown residue fluorescing almost a quinine blue when diluted with alcohol containing water to a faint yellow colour. It is relatively insoluble in water in acid solution, but dissolves in quantity upon the addition of alkali. It has not been possible so far to separate the blue fluorescing substance from the pigmented matter. Weight for weight the crude fraction is at least five times more powerful than quinine sulphate as a source of blue fluorescence. Porphyrin is present in this fraction, and can be extracted from it by the method described by Fischer [1923] for the extraction of Kammerer's porphyrin, after solution in ether containing acetic acid. After removal of the porphyrin there are no bands seen in the extracts of fluorescent material, merely a vague absorption up to λ 490.

By treatment of the aqueous phase fractions obtained in method 1 with alcoholic potash, and subsequent shaking with ether, an ethereal extract is obtained which fluoresces green, changing to blue on neutralisation.

[1] Dr Howitt mentioned to us that he has found blue fluorescence in human serum We therefore examined rabbit serum and found fluorescence. The matter is being further investigated.

(*b*) *Urine.*

All the urines examined fluoresced a decided blue though this tint is slightly modified by the green fluorescence of urobilin. The urochrome fraction prepared by the Garrod technique [1894] fluoresces blue when diluted to a pale yellow.

As in guano, there are present two classes of fluorescent fractions, distinguished by their behaviour on fractionation from a water-alcohol-ether mixture. There is relatively more of the aqueous fraction present in the urines examined by us than in the guano extracts. The behaviour of the aqueous fraction on saponification with alcoholic potash is similar to that of the guano fraction.

(*c*) *Bile.*

Bile was treated with acetone and the precipitate removed. When this was shaken with ether in successive portions a deep violet blue fluorescence appeared in the ether phase quite different from the blue fluorescence previously mentioned. We obtained some evidence that there was also a "blue fluorescent substance" present in the aqueous layer, but it is difficult to see, as it is much obscured by bile pigment. It will be noted from Table I that specimens of cholalic and cholic acids showed no fluorescence. Laboratory specimens of Na taurocholate and glycocholate gave a slight blue fluorescence, quantitatively of a different order from the guano extracts. This appeared to be due to an impurity, as, in the glycocholate, the blue fluorescence passed readily into a chloroform layer upon shaking the aqueous solution with chloroform.

2. *Other animal extracts.*

We have obtained chloroform extracts fluorescing blue from sheep's liver, milk, and white hen feathers by extraction with acid alcohol followed by treatment with chloroform, and also from caseinogen by extraction with 95 % alcohol. Blood plasma and serum when diluted showed no fluorescence.

The extracts obtained under (1) and (2) are very similar in type. We have obtained evidence of a different behaviour in the case of (3).

3. (*a*) *Gelatin.*

Commercial leaf gelatin in the solid state and when dissolved in water fluoresces blue. The fluorescence of the solid gelatin is intensified by soaking in acetone. The blue fluorescence is hardly affected by boiling with dilute acids, but disappears in large part after treatment with alcoholic potash. We have not been able to obtain by any means an ether-alcohol extract from gelatin, which fluoresces blue. Further samples of flocculated gelatin kindly supplied by Prof. Schryver fluoresced a faint blue, but much less intensely than the commercial gelatin.

(*b*) *Skin.*

The skin used came from the calf and was freed from subcutaneous tissue as far as possible. Pieces of skin some 12 inches square were used for the experiments. The skin fluoresced an intense quinine blue when examined in the ultra-violet light.

Exp. 1. Skin was heated with 50 % alcohol containing 1 % HCl. None of the fluorescing material was extracted. After standing for some days the alcoholic extract was removed and the skin treated with alcoholic potash for 15 minutes. The mixture was neutralised with acetic acid and HCl. By extraction of the neutral extract with ether, blue fluorescence was seen in the ether layer, but the greater part remained in the aqueous phase.

Exp. 2. Skin was hydrolysed with alcoholic potash as a first step. The hydrolysate was evaporated nearly to dryness on the water bath. An aqueous solution of the residue fluoresced blue. When the alkaline solution was shaken with ether, there was seen a yellowish blue fluorescence in the ether layer. After neutralisation of the solution with acetic acid, the ethereal layer fluoresced blue.

Exps. 1 and 2 indicate that though there is present in skin some blue fluorescent substance which will pass into ether, there is also a large amount of material which will not pass into an ether layer. Exp. 3 shows that this substance can be extracted with dilute acid.

Exp. 3. Skin was heated with dilute aqueous HCl in an attempt to follow the technique of Bence Jones. In this way an extract of a gelatin type was obtained which fluoresced an intense blue, and was readily thrown out of solution by alcohol. The acid extract was concentrated and treated with acetone to dry it. It was then extracted under the reflux condenser with boiling absolute alcohol and ether successively. Neither of these extracts fluoresced. After removal of the ether, the fluorescent residue was hydrolysed with 5 % HCl for some hours. After this the fluorescence was still present though it seemed to be diminished in intensity. Upon neutralisation, again no fluorescent material could be extracted by ether. The acid hydrolysate was neutralised and treated with alcoholic potash, after which treatment again no fluorescent ethereal extract could be obtained.

From these experiments we conclude that though there are present traces of substances in the skin which will yield "blue fluorescent" ether extracts, the main part of the blue fluorescence of skin is due to some other constituent in which fluorescence is associated with a gelatin-like substance.

(*c*) *Lens.*

The substance causing blue fluorescence of the lens of the sheep and ox could not be extracted with acid 50 % alcohol, and behaved similarly to the acid aqueous extract of skin in that it could not be extracted by ether, and was largely destroyed upon saponification with alcoholic potash.

C. *Plant Products.*

(1) *Grass.*

Grass leaves were treated as described by Plimmer according to the Willstätter technique for the preparation of chlorophyll, xanthophyll and carotene. None of these extracts fluoresced blue; chlorophyll was purplish red, xanthophyll yellow and carotene greenish yellow when examined under our experimental conditions. An extract of carotene prepared from carrots behaved similarly. We found, however, that the first acetone extract of the leaves which is usually discarded in the preparation of chlorophyll, after thorough extraction with light petroleum to remove chlorophyll, fluoresced a quinine blue.

(2) *Oils.*

Certain oils were examined and were found to fluoresce blue. Cod liver oil gave a greenish yellow fluorescence. Table II shows an approximate comparison of the strength of blue fluorescence found in oils purchased locally.

Table II.

Oil	Dilution in ether fluorescing blue with intensity of 0·05 % quinine sulphate
Cotton seed	1 in 18
Linseed	1 in 18
Olive	1 in 3
Colza	1 in 5

In the case of the cotton seed oil it was found that the fluorescent constituent was present in the unsaponifiable fraction after removal of the main part of the cholesterol by digitonin. The others were not examined in this respect.

(3) *Other products.*

An extract prepared by Mr Peskett from cane sugar in the course of the preparation of bios-free cane sugar fluoresced blue.

It was found that a blue fluorescent extract could be obtained from sawdust by treatment with acid 50 % alcohol followed by addition of chloroform.

As mentioned in the introduction, yeast concentrates obtained by the charcoal method described elsewhere [Peters, 1924] fluoresced a quinine blue.

Some remarks upon the nature of the substances involved in the blue fluorescence.

In the main we confirm Bence Jones in that most animal tissues contain substances extractable in ether-alcohol mixtures which fluoresce blue under our conditions. We may call these "animal" quinoidine after Bence Jones, of which typical examples are the guano and urochrome fractions. The substance present passes into an alcohol-ether layer either directly or after saponification with alcoholic potash. There is however some difference in the behaviour of the fluorescent substance in gelatin, skin and the lens, which we may designate

quinoidine "S." Fluorescence largely disappears upon saponification with alcoholic potash, and further cannot be made to pass into an ether layer. Whether this constitutes a real difference or depends upon some accidental association is not clear at present. The absence of blue fluorescence from blood plasma and serum[1], and also from egg albumin, shows that this fluorescence is not an inherent property of proteins, and the fact that crude gelatin fluoresces more powerfully than flocculated gelatin suggests that fluorescence of gelatin is due to an impurity.

The quinoidine fractions from urine and guano are very similar in appearance and behaviour. We think that the following arguments prove that blue fluorescence is not due to the presence of porphyrins: (1) As mentioned above, the traces of porphyrin present can be removed from the guano ether extracts without interfering with their blue fluorescence. (2) In the case of urine, porphyrins were shown to be present in normal urine in traces by Garrod [1893]. Owing to the work of H. Fischer and his colleagues we have to consider as possibilities uro-, copro- and Kammerer's porphyrin. We have never found that these porphyrins fluoresce blue. Specimens of uroporphyrin prepared by one of us from the urine of "Levi," a case of haematoporphyria congenita, by the methyl ester method of Fischer and Zerwecke [1924] fluoresce an intense red. Crude specimens of coproporphyrin show a yellowish red fluorescence. Turacin, believed by Fischer to be the copper salt of uroporphyrin did not fluoresce under our conditions. (3) The plant porphyrin fractions obtained by us showed no blue fluorescence. (4) The blue fluorescence is not due to the interaction of porphyrin and some other constituent because the addition of uroporphyrin to normal urine produces no intensification of the blue fluorescence, merely a change of hue.

Quinoidine extracts from guano have been obtained which gave no trace of Whitby's [1923] reaction for sterols with formaldehyde and sulphuric acid, and no blue colour with sulphuric acid alone, excluding sterols and lipochromes. Cholesterol itself was excluded by direct test of the pure substance (Table I). The Schlesinger test for urobilin was negative, also the Hymans van den Burgh reaction. Positive indican and kynurenic acid tests could not be obtained.

We feel that it is most probable that blue fluorescence may prove to be actually a property of urochrome for the following reasons. Firstly, the uroquinoidine extracts from guano are at least five times more fluorescent than quinine. If the fluorescence be merely due to contamination, there must be present a substance which is extraordinarily powerful as a source of blue fluorescence. Secondly, the behaviour of the aqueous fractions from guano and urine after saponification with alcoholic potash suggests the decomposition of some complex, which may quite possibly be of the glycuronic acid type. This would be consistent with the recent finding of Pollecoff [1924] that glycuronic acid is a constituent of urochrome. Two views have been

[1] See note, p. 406.

taken about the origin of urochrome. The first is that of Roaf [1921] who considers that it is a derivative of chlorophyll, as he found that feeding upon the green parts of plants increases the amount of the urochrome fraction in the urine. The second is the view of Fischer and Zerwecke [1924], who have found evidence for the presence of certain amino acids in urochrome, and consider it to be a breakdown product of haemoglobin. Our evidence, based upon blue fluorescence, would suggest that urochrome comes from the green parts of plants, not from chlorophyll, but from some "blue fluorescent" substance present in the first acetone extract of the leaves.

The fluorescence of "animal quinoidine" is fairly resistant to oxidation, but readily disappears upon treatment with acid solutions of nitrite.

Discussion.

There are two questions involved in this work. Firstly, do these substances fluoresce blue because of the possession of some common constituent? Further work will doubtless settle this point. Secondly, there is the broader biological question. What is the meaning of this widespread blue fluorescence which we find in animal epithelial structures, in the lens of the eye, in yeast, in sawdust and in grass? Whenever ultra-violet light falls upon these parts it is certain that they must be exhibiting this blue fluorescence. Up to the present it has been considered that the presence of fluorescent substances in the skin is dangerous to the animal organism. This view is based upon the experiments of Raab [1900], Tappeiner [1900], Hausmann [1911] and others upon the toxic effect of fluorescent substances, and upon the blistering found to occur in cases of haematoporphyria in sunlight. Are we to believe that, whereas the presence of a red fluorescence is dangerous, the presence of a blue fluorescence is not? In this connection the experiments of Suarez [1916] are of interest, who obtained from maize a substance resembling quinine in fluorescence, and called by Hofmeister "Zeochin." This zeochin sensitised blood corpuscles and organisms to the light, but when fed to animals in large amounts it did not render them light sensitive, thus suggesting that, normally, the animal can deal with such substances.

If "quinoidine" should turn out to be identical with urochrome, it would be of interest in connection with the work of Fischer and Zerwecke [1924]. They found that urochrome protected paramoecia against the toxic action of porphyrins in the light. It is difficult to believe that there is not some real function in this interaction of ultra-violet light with "quinoidine S." Recent experiments by Hess and Weinstock [1925], Steenbock, Black, Nelson, Nelson and Hoffert [1925] and Coward and Drummond [1925] show that irradiated cholesterol has antirachitic effect so that the presence of fluorescence cannot be essential to the formation of antirachitic substance. It may, however, be in some way directive. There is a "blue fluorescent" substance present in milk, sawdust and certain oils. Further, cotton seed and linseed oil are some

six times more fluorescent than olive oil. It may be mere coincidence, but the facts seem to be related to the findings of Hess and Weinstock [1924] and Steenbock and Black [1924] that irradiated cotton seed and linseed oils give good cures whereas olive oil gives inconstant results.

Summary.

1. The blue fluorescence of tissues in ultra-violet light (Å. 3000–4000) has been investigated qualitatively. Extracts fluorescing blue can be obtained, as was shown originally by Bence Jones who called the substance responsible "animal quinoidine."

2. Quinoidine extracts can be obtained from urine, guano, milk, feathers, liver and caseinogen, also from skin, commercial gelatin and the lens of the eye. The behaviour of the latter group to solvents is different. Whether this difference is the accident of some association is not yet clear.

3. Phytoquinoidines are present in grass leaves and certain oils, especially linseed and cotton seed, in yeast extracts and also in sawdust.

4. No pure substance among a number examined showed a quinine-like fluorescence. The fluorescence of porphyrins, chlorophyll, xanthophyll, and bile derivatives was not blue.

5. Upon fractionation of normal urine the quinoidine passes into the urochrome fraction (Garrod).

We are indebted to Sir Archibald Garrod and Dr Mackey for an introduction to the rare case of haematoporphyria congenita, to Professor Goodrich for turacin and to Sir F. G. Hopkins for a specimen of histidine. We are also most grateful to Professor Schryver for his kindness in sending specimens of flocculated gelatin. One of us (H. W. K.) is indebted to the Medical Research Council for a grant.

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LX. THE PECTIN CONTENT OF NORMAL AND "SILVERED" APPLE LEAVES.

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(*Received March 30th, 1925.*)

During a recent investigation the observation was made that the young bark of the apple contained a very appreciable amount of pectin. The variety of apple examined was Bramley's Seedling. The bark, after mincing, was thoroughly extracted with boiling alcohol and then, after drying, with boiling water. The aqueous extracts, after concentration under diminished pressure, yielded, on precipitation with alcohol, a product which appeared to be essentially identical with the pectin obtained from apple fruit, with the exception that it contained a very small amount of starch. On treatment with alkali it gave methyl alcohol and acetone and on treating its aqueous solution with the enzyme, pectase, a good gel was soon formed, although not quite so quickly as was the case when employing pectin obtained from the fruit.

This discovery of pectin in the young bark suggested that apple leaves might also contain this substance. A quantity of apple leaves was therefore examined for pectin in a manner similar to that employed when investigating the bark. A good yield of pectin was then obtained, but it was not quite so pure as that derived from the bark since it contained rather more starch and did not gel so quickly with pectase.

It was observed, however, that while the leaf fragments, which had been previously exhausted with boiling alcohol, were being deprived of pectin by means of boiling water they developed a silvery appearance precisely resembling that shown by leaves affected by the well-known "Silver Leaf" disease. The possibility of "Silver Leaf" being associated with pectin deficiency was therefore investigated.

As is well known, "Silver Leaf" is a disease particularly affecting plum trees, but other trees, including the apple, may also become infected by it. Leaves of the last mentioned were chosen for the present investigation since material particularly well adapted for this purpose was readily available, and, moreover, the results so obtained would be comparable with the observations already made, which are recorded above.

The tree chosen was an espalier apple of the "Keswick Codlin" variety, the upper branch on one side of which only was affected with "Silver Leaf"

disease. On the morning of July 22nd 40 average leaves, without stalks, were collected from near the end of the diseased branch, and a like number from a similar position on the corresponding branch on the other side of the tree, which was perfectly normal. After weighing, each sample of leaves was cut into narrow strips and thrown into boiling alcohol. After boiling for 15 minutes the extractions were completed in a Soxhlet apparatus with fresh quantities of alcohol. After drying and weighing the material which had been exhausted with alcohol, each sample was extracted a number of times by boiling with large volumes of distilled water for the removal of the pectin. The latter, after concentration of the extracts under diminished pressure, was precipitated by means of alcohol, collected, dried and weighed. The results obtained are given below:

	Original weight of 40 leaves g.	Weight of alcohol-extracted marc g.	Weight of pectin g.	% of pectin in alcohol-extracted marc
Normal leaves	35·15	7·70	1·30	16·89
"Silvered" leaves	29·70	6·50	0·97	14·90

It is thus seen that the leaves affected by "Silver Leaf" disease contain an appreciably smaller amount of pectin than do normal leaves. Moreover, the yield of pectin calculated on the weight of the fresh, normal leaves (3·7 %) seems surprisingly large when compared with the proportion of this substance found by the present author [1921, 1923] to occur in the peel and pulp of the fresh fruit, namely, 2·6 and 1·3 % respectively. The pectin obtained from the leaves, however, is not so pure as that prepared from the fruit, but the amount of impurity present certainly does not seem sufficient to account for the increased yield obtained from the leaves as compared with that from the fruit.

Although the presence of pectin in apple leaves does not appear previously to have been recorded, it is known that they contain the enzyme, pectase. Thus, if fresh apple leaves be well pounded with a little water and the resulting mass strongly pressed, the juice so obtained, if added to a solution of pectin in the presence of chalk, causes the formation of a solid gel of calcium pectate. The expressed juice of the leaves appears, however, to contain little, if any, pectin.

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LXI. CHEMICAL INVESTIGATIONS OF FRUITS AND THEIR PRODUCTS.

I. APPLE JUICE AS A SOURCE OF SORBITOL.

By FRANK TUTIN.

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(*Received March 30th, 1925.*)

The material generally employed as a source of the hexahydric alcohol, sorbitol, namely, the berries of the Mountain Ash (*Pyrus aucuparia*, Gaertn.), is not always readily obtainable, even at the appropriate season of the year. Moreover, in the author's experience, it is by no means always an easy matter to isolate sorbitol from this source. During an investigation on the constituents of apples it has been found that they contain an appreciable proportion of sorbitol and, since apples are always obtainable and the isolation of sorbitol from them may easily be accomplished, it would appear that this fruit affords the most convenient source for the compound in question. The method employed for the isolation of sorbitol from apples is briefly as follows.

The apple juice was first concentrated under diminished pressure to about one-third its volume. A slight excess of basic lead acetate solution was then added and the resulting precipitate removed by filtration. The filtrate, after being deprived of lead by means of hydrogen sulphide, and the excess of the latter removed, was fermented with yeast in an incubator until the evolution of carbon dioxide had apparently ceased[1]. The mixture was then considerably concentrated under diminished pressure and again treated with basic lead acetate as previously described, after which the filtrate, which was now much diminished in volume, was again fermented, as it was found that an appreciable amount of sugar always remained after the first fermentation was apparently complete. A final clearing with lead was then employed and the filtrate, after being deprived of lead, was evaporated as far as possible under diminished pressure.

The gum-like residue so obtained was then covered with an excess of acetic anhydride, some pyridine added as a catalyst, and the mixture cautiously warmed on a water-bath, when acetylation occurred rapidly after the temperature had been somewhat raised. When the first vigorous reaction was over the mixture was kept boiling for 1 hour to ensure complete acetylation, after

[1] The addition of some "yeast water" and a little ammonium phosphate greatly accelerates the fermentation.

which it was cooled to about 50° and water cautiously added to decompose the excess of acetic anhydride present. Finally, a considerable excess of water was added and the mixture extracted several times with ether. The combined ethereal extracts were washed several times with water and then extracted successively with aqueous sodium carbonate and sodium hydroxide, after which they were again washed with water, and dried. During the extraction with alkalis and the final washing with water appreciable amounts of brown, resinous products were removed. On evaporating nearly all of the solvent from the purified ethereal extract a pale yellow residue was obtained, which soon crystallised in aggregates of hard, colourless prisms. The latter were deprived of adhering, pale yellow, viscid material by draining on a porous tile in an atmosphere saturated with ether. After recrystallisation from ethyl acetate colourless prisms melting sharply at 99° were obtained, and were identified as hexa-acetylsorbitol. In *Richter's Lexicon* the latter compound is described as a syrup, on the authority of Vincent and Delachanal [1889] but Thorpe [1912] gives the melting point as 99°, in accordance with the value found by the present author. Hexa-acetylsorbitol is readily soluble in alcohol, moderately so in ethyl acetate and rather sparingly soluble in ether. It dissolves slightly in boiling water.

For the regeneration of sorbitol 2 % aqueous sulphuric acid was added to a quantity of the above-described hexa-acetyl derivative and the mixture boiled in a flask attached to a reflux condenser until solution was complete. Steam was then passed through the mixture until acetic acid was no longer removed, after which the sulphuric acid was exactly precipitated by the addition of aqueous baryta, and the filtrate from the barium sulphate evaporated to dryness under diminished pressure. The residue so obtained was dissolved in alcohol from which it slowly separated in colourless crystals which, after drying, melted at 110°, and were identified as *d*-sorbitol.

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LXII. CHEMICAL INVESTIGATIONS OF FRUITS AND THEIR PRODUCTS.

II. THE FATE OF SUGAR DURING "CIDER SICKNESS."

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(*Received March 30th, 1925.*)

The disease known as "Cider Sickness" or "Second Fermentation" is, amongst all the disorders to which cider is liable, the most dreaded by cider makers in this country. It has been much studied by Barker [1906, 1907, 1911, 1912, 1, 2] who has shown that it is caused by a specific organism which attacks chiefly sweet ciders of low acidity when the temperature becomes somewhat raised. It causes a very rapid disappearance of the sugar, together with the formation of alcohol, acetaldehyde, formaldehyde, carbon dioxide, and hydrogen. He also obtained conclusive evidence that part of the sugar was converted into some non-reducing substance, which could not then be isolated nor its nature ascertained.

Since the acetylation method described in the preceding paper had proved so convenient for the isolation of sorbitol from apple juice it was decided to employ it for the investigation of the non-reducing substances present in "sick" cider. A quantity of the latter in which the "sickness" fermentation had been allowed to proceed to the ultimate limit was therefore concentrated under diminished pressure, purified by means of basic lead acetate, and the solids contained in the filtrate acetylated in the manner described in the foregoing paper. The resulting acetylated material, after purification in the manner employed when isolating the hexa-acetylsorbitol (preceding paper, p. 417), was obtained in noticeably greater yield than the corresponding product from the fresh juice. It could not, however, be induced to crystallise either by long keeping or by inoculating with a crystal of hexa-acetylsorbitol. When, however, a small particle of hexa-acetyl-*d*-mannitol was introduced crystallisation commenced and, during the course of some weeks, gradually spread throughout the viscid mass, but the proportion of crystalline material deposited was not large. The very viscid mixture was then brought on to a porous tile and allowed to drain in an atmosphere saturated with alcohol vapour. The white solid so obtained, after one crystallisation from alcohol, melted at 123° and was identified as hexa-acetyl-*d*-mannitol. A small amount of hexa-acetylsorbitol

was subsequently isolated from the viscid material which had been absorbed by the tile.

Since no mannitol could be detected in apple juice after fermentation with yeast only, it appears evident that fermentation by the "sickness" bacillus results in the reduction of a portion of the sugar present to mannitol, a change which is known to be brought about by certain other bacteria and which is in harmony with the observation of Barker that hydrogen is evolved during "sickness" fermentation.

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LXIII. OXIDISING ENZYMES.

VIII. THE OXIDATION OF CERTAIN PARAHYDROXY-COMPOUNDS BY PLANT ENZYMES AND ITS CONNECTION WITH "TYROSINASE."

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(*Received April 1st, 1925.*)

It has been shown by one of us [Onslow, M. W., 1920] that the oxidases of the higher plants may be represented as a system consisting of two enzymes (oxygenase and peroxidase) and a substance having the orthodihydroxy-grouping of catechol. The oxygenase catalyses the autoxidation of catechol with formation of peroxide; the peroxidase catalyses the transference of active oxygen to guaiacum. Substances having the catechol grouping, for instance, catechol, protocatechuic acid, caffeic acid, hydrocaffeic acid and chlorogenic acid [Gorter, 1909] have been detected by various workers in a number of plants, all of which (in cases where tested) have been oxidase plants.

It was also shown [Onslow, M. W., 1920] that, in addition to substances with the catechol grouping, the widely distributed oxygenase will catalyse the oxidation of *p*-cresol with the resultant blueing of guaiacum. With this exception, however, the oxygenase was unable to act on various phenols with groupings other than catechol, for instance, phloroglucinol, resorcinol, etc. It was suggested [Onslow, M. W., 1923] that this behaviour of *p*-cresol might be explained by the presence of traces of a dihydroxy-derivative formed either by autoxidation or by traces of active oxygen in the enzyme preparation.

The present communication records the fact that when an enzyme preparation from the potato tuber acts on either *p*-cresol, tyrosol (*p*-hydroxyphenylethyl alcohol) or phenol a dihydroxy-derivative is formed which gives rise to peroxide and subsequent blueing of guaiacum.

The potato tuber, as previously shown, contains oxygenase, peroxidase and a substance with the catechol grouping. It also contains "tyrosinase" and free tyrosine or polypeptides of tyrosine, or both.

An enzyme preparation of the tuber was made by thoroughly extracting with cold alcohol, and then extracting the residue with water. When added to *p*-cresol solution, the mixture rapidly turned orange-pink. On addition of guaiacum to a portion, a deep blue colour was produced. On addition, to

another portion, of a few drops of a weak solution of ferrous sulphate or ferrous ammonium sulphate, the pink colour turned green and subsequently with 1–2 % sodium carbonate blue, violet and red, a reaction characteristic of the catechol grouping. When the enzyme was added to tyrosol, a pink colour was rapidly developed. The solution then gave the same reactions as those of *p*-cresol with guaiacum and with ferrous sulphate and sodium carbonate. In the early (red) stage of the action of the enzyme on tyrosine the iron solution appeared to give the catechol reaction, but it was not normal.

The above results indicate a new type of oxidation. The solutions of *p*-cresol and tyrosol may contain traces of a dihydroxy-compound formed by autoxidation, too small in amount to be detected by the iron reaction[1]. Should this be so, the enzyme may be regarded as catalysing this autoxidation by means of active oxygen. An alternative suggestion is that the enzyme preparation is never quite free from traces of active oxygen which may produce traces of the dihydroxy-compound [Dakin and Herter, 1907] thus enabling the reaction to proceed. A third suggestion is that an enzyme exists which has a specific action on parahydroxy-compounds, and the same distribution as oxygenase.

An enzyme preparation from the tuber was also added to a solution of dihydroxyphenylalanine (prepared from *Vicia faba*). A red colour was first formed which eventually became black as in the case of tyrosine. Addition of guaiacum at the red stage did not produce more than a trace of blue colour on the surface of the liquid. It is well known, however, that the products of "tyrosinase" on tyrosine do not blue guaiacum.

It has been shown collectively by Chodat and Schweizer [1913], Happold and Raper [1925] and Robinson and McCance [1925] that *p*-cresol, catechol and phenol, in presence of an enzyme preparation from the potato tuber, will oxidise glycine, alanine and leucine.

One of us (M. E. R.) has also found that potato enzyme and tyrosol will oxidise glycine. Tyrosine and dihydroxyphenylalanine will not, however, effect the oxidation of glycine.

The conclusions which we draw from the above facts are as follows:

The oxygenase (or some other enzyme) oxidises *p*-cresol and tyrosol to 3.4-dihydroxytoluene and 3.4-dihydroxyphenylethyl alcohol respectively. These then give rise to active oxygen in the same way as catechol.

The active oxygen is the agent in the deamination of glycine, alanine and leucine.

In the case of dihydroxyphenylalanine, active oxygen formed may be used in deamination of the side chain. Hence there is no blueing of guaiacum. Dihydroxyphenylacetaldehyde and ammonia formed may take part in melanin formation.

[1] Against this view in the case of *p*-cresol is the fact that a solution of this substance, on standing in air, does not give a blue colour with horse-radish peroxidase and guaiacum as does a solution of catechol kept under similar conditions.

Tyrosine may be oxidised (in the same way as tyrosol and *p*-cresol) to dihydroxyphenylalanine, the reaction proceeding subsequently as above.

On the other hand, Raper and Wormall [1925] maintain that deamination of tyrosine does not take place, since no ammonia can be detected during oxidation. In addition, they have stated that the enzyme has no action on *p*-hydroxyphenylpyruvic acid in the presence of ammonia.

It has been shown by Bach [1914] that the preliminary red colour formed in the oxidation of tyrosine by "tyrosinase" disappears if the products are placed under anaerobic conditions. Hence Bach postulated the presence in the potato of an oxido-reductase (deaminase) which forms products which are eventually oxidised to melanin either spontaneously or through the accelerating influence of a phenolase.

It has been suggested by one of us [Onslow, 1923] that the oxido-reductase may act as an intermediary between the active oxygen and the amino-acid.

Raper and Wormall [1923] doubt the existence of an oxido-reductase for the following reasons. Potato "tyrosinase" (dialysed juice) was allowed to act on tyrosine until a red colour was produced. The solution was divided into two portions. From one portion the enzymes were removed by colloidal "iron," leaving a red solution. Both portions were then placed in an incubator at 38°, and they decolorised equally well.

We noted, as did these authors, that the red oxidation product of "tyrosinase" on tyrosine was decolorised on heating. So also, to a lesser extent, was the red-brown product formed by the action of oxygenase on catechol. The same was true for the blue oxidation product of guaiacum. A similar experiment was made by us as follows. An enzyme preparation of the potato tuber was prepared by reprecipitation with alcohol so as to remove amino-acids as far as possible. This was allowed to act on catechol until a red-brown product was formed. Half the solution was treated with colloidal "iron." The two solutions, with and without enzyme, were then evacuated in Thunberg tubes and left at room temperature. The tube containing enzyme decolorised in 3 hours. The control tube remained coloured for one or more days. We do not necessarily conclude that an oxido-reductase is present, since the enzyme preparation would certainly contain proteolytic enzymes, and oxidised catechol is undoubtedly decolorised by products of autolysis.

Further work is in progress to determine, if possible, whether or no an oxido-reductase is present which catalyses the oxidation of the amino-acids present.

We conclude from the evidence available that the so-called action of "tyrosinase" may be as follows. Oxygenase (or some other enzyme) introduces a second hydroxy-grouping forming dihydroxyphenylalanine. Oxygenase catalyses the oxidation of this substance to a peroxide. Active oxygen may oxidise the amino grouping directly or may act as an acceptor for the hydrogen produced by an oxido-reductase, the amino-acid acting as the acceptor for the oxygen.

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Note added at proof reading, May 21*st*, 1925. We have found that *m*-cresol (both Merck and Kahlbaum samples) is oxidised by the enzyme preparation to a compound giving the catechol reaction. A 1–2 % solution of *p*-hydroxybenzoic acid, neutralised with sodium hydroxide to litmus, is also slowly oxidised by the enzyme, and the solution, after 24 hours, gives a slight but distinct catechol reaction.

LXIV. CHOLESTEROL SYNTHESIS IN THE ANIMAL BODY.

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(*Received April 2nd, 1925.*)

Ellis and Gardner [1909] attempted to determine whether cholesterol synthesis occurred in the animal body by determining the amount of cholesterol in a number of unincubated eggs and in newly-born chicks from the same batch of eggs. They found that the amount in the newly-born chicks did not differ from that in the eggs, and they concluded that cholesterol synthesis did not occur. They laboured however under the difficulty that they were using a method of cholesterol estimation much less accurate than that available at the present time. Gardner and Lander [1914] approached the question from another point of view. They estimated the cholesterol in a number of newly-born chicks and fed the remainder of these chicks on various diets—chicken food, chicken food previously freed from sterols, and chicken food plus 2 % cholesterol, and, after 7, 14 and 21 days, further estimations were made on individuals from the various groups. Owing to irregular rate of growth and consequent irregular weights, the results were not very definite, but the authors conclude that cholesterol synthesis does not take place. Lander [1915], carrying out similar experiments on rats, found that the percentage cholesterol was, on the average, 161 mg. at 3 weeks of age. He fed other rats on mixed, and in some cases ether-extracted, diets, but none of his animals lived for any length of time owing to the fact that his diets were deficient in vitamin A. His inconclusive results are therefore open to objection, but he expresses the view that "there is no evidence that the living organism can manufacture cholesterol, but on the other hand, it is strictly conserved and readily picked out from the diet." His method of extracting the cholesterol was to mince the animals, grind them up with plaster of Paris, and extract the powdered material for 14 days with ether. Cholesterol was estimated in the extract by the digitonin method.

The work so far described has had as its basis the estimation of cholesterol in the whole body of the animals under experiment. Another method has been to measure the total sterol intake on mixed diets and the total excretion in the faeces. Thus Gardner and Fox [1921], working with eight adults on mixed diets, found that the faecal sterol output was about 2·5 times greater than the

intake. In all cases save one this large excess excretion over intake was maintained, and they conclude that there is some organ in the body capable of synthesising cholesterol. Similar results are reported by Gamble and Blackfan [1920] in which the sterol output was 1·3–3·4 times the intake. These concordant results lead the authors to conclude that cholesterol synthesis takes place, but there are certain facts which this type of experiment ignores. Thus, even though the sterol output is greater than the intake, no rigid conclusion as to cholesterol synthesis can be drawn unless knowledge exists as to the function of the cholesterol stored in the various organs of the body. Little is known on this subject and the excretion of cholesterol might possibly ensue as the result of some physiological function; in this case if the diet were poor in cholesterol, the reserve supplies in the liver, adrenals, etc., might be called upon to make up the deficiency, and hence, unless experiments of this type were carried out over a prolonged period, the possibility of the effect being due to cholesterol already stored in the body cannot be eliminated. A second objection is that bacterial synthesis of sterol in the large intestine may be the cause of the large excess of excretion over intake. It was with a view to investigating these possible sources of error that the work described in this paper was undertaken. The general plan was to estimate the amount of cholesterol in rats at various ages and weights when on the complete artificial diet suggested by Drummond and Coward [1920] and used in this laboratory, in others on a diet poor in cholesterol, and in others still on a diet completely free from cholesterol.

The difficulty with regard to the cholesterol-free diet has hitherto been that no diet has been devised which is free from cholesterol and yet sufficient for growth. The ether-extracted diets used by other workers have necessarily been free from fat and therefore from fat-soluble vitamins. Hence where such diets have been used the results have suffered from the fact that growth, if any, has been very slight, and in experiments of this type it would appear essential that the animals should exhibit normal growth throughout. This difficulty has been overcome in the present work by supplying the rats with a very small daily dose of the unsaponifiable matter from cod liver oil. This is prepared by extracting cod liver oil, after saponification with alcoholic potash, with ether. After washing the extract and removing the ether, the resulting product, which still contains about 5 % of oil, is again saponified and extracted with ether as before. The washed extract, after drying over anhydrous Na_2SO_4, is taken to dryness. The unsaponifiable matter so obtained contains about half its weight of cholesterol, the bulk of which can be removed by crystallisation from 95 % methyl alcohol. After filtration and removal of the solvent, the residue is dissolved in boiling 95 % ethyl alcohol and the remaining cholesterol precipitated by excess of a 1 % solution of digitonin in 90 % alcohol. After filtration and removal of the excess digitonin, the solvent is evaporated off. Suitable precautions are taken to prevent oxidation throughout the preparation.

Diets used in the experiment.

I. Complete artificial diet	%	II. Cholesterol-free diet	%	III. Diet poor in cholesterol	%
Caseinogen	20	Caseinogen (ether extracted)	20	Caseinogen (ether extracted)	20
Rice starch	50	Rice starch	68	Rice starch	68
Butter	15	Butter	0	Butter	0
Yeast extract	5	Yeast extract	7	Yeast extract	7
Lemon juice	5	Lemon juice	0	Lemon juice	0
Salt mixture	5	Salt mixture	5	Salt mixture	5
		Unsap. matter (cholesterol-free)	0·0005 g. per rat per day	Unsap. matter	0·0005 g. per rat per day
				Cholesterol	0·004 g.

With a view to economising the unsaponifiable matter, it was dissolved in synthetic triolein and each animal of the group on the cholesterol-free diet received one drop of this solution per day, this being made up so that one drop contained approximately 0·0005 g. unsaponifiable matter. The cholesterol of diet III was given in a similar manner.

Fat and cholesterol content of the diets.

In the cholesterol-free diet (II), the fat (butter) was omitted and the starch increased to 68 %. The lemon juice was also omitted, as rats appear to be able to grow satisfactorily on a diet deficient in vitamin C. In order to render the diet more palatable, the percentage of yeast extract was increased to 7. As the caseinogen used had been thoroughly extracted, this diet was virtually fat-free. To obtain its fat content, 25 g., after grinding with anhydrous Na_2SO_4 were extracted with ether for 40 hours, and yielded 0·2 % ether-soluble material. This was saponified with sodium ethylate and the resulting unsaponifiable matter gave no precipitate with digitonin and none of the cholesterol colour reactions. As further confirmation that the diet was quite free from cholesterol, 25 g. were saponified first with aqueous and then with alcoholic potash. The unsaponifiable matter obtained by ether extraction also failed to give any of the cholesterol colour reactions; this diet was thus cholesterol-free, and constituted the basis of diets II and III. In diet III, each animal received three drops of a 4 % solution of cholesterol in triolein, thus receiving approximately 4 mg. cholesterol per day.

Diet	Basal fat g. per rat per day	Fat in supplement g. per rat per day	Total fat g. per rat per day	Total cholesterol g. per rat per day
I. Complete artificial	5·5	—	—	0·0015
II. Cholesterol-free	0·02	0·025	0·045	—
III. Poor in cholesterol	0·02	0·075	0·095	0·004

Animals used in the experiment.

All the rats used in this work were bred from a definite number of our own stock, with a view to eliminating abnormal results as far as possible. The original plan was to estimate the cholesterol content of rats at time of weaning

(age, 3–4 weeks, weight, 35–45 g.) and to take an average of the figures obtained as representing the normal cholesterol content of rats at this age and weight. Any animals put on to any diet at weaning were thus considered as starting with this average amount of cholesterol. Later it was decided to do estimations on numbers of rats on the complete artificial diet (diet I) at various ages and weights—in other words to obtain figures for a curve of cholesterol content of rats on this diet plotted against age or weight. This was to serve as a control of the results obtained from animals on the other diets. Every group of animals on any given diet was a mixture from the various litters, care being taken that the litters were well scattered among the different groups in order to obtain more average results.

Method of estimation.

Each rat was killed by chloroform; its alimentary canal was removed from the stomach downwards and washed through with water until quite empty. In this way only the cholesterol present in the tissues was estimated. The animal, together with the washed stomach, intestine, etc., was then placed in a flask with sufficient 10 % KOH and boiled for 3–5 hours, when complete solution, save of the bone fragments, had taken place. It was considered that in this way only could all the cholesterol be freed from the tissues. The results, which will be reported later, show a higher cholesterol content than those of Lander [1915] for animals of the same age and weight. This would suggest that either the cholesterol was not completely extracted by his method or else that his animals were considerably under the weight which those reared in this laboratory normally show at the same age. After saponification by aqueous KOH, the liquid was extracted with ether six times in a separating funnel after addition of sufficient alcohol to cause rapid separation of the ether and aqueous layers. The extract—usually about 500 cc.—was washed five times with water, and after removal of the ether the resulting fat was dried at 105° for half an hour. It was then boiled for 2 hours with sodium ethylate (2*N*) after solution in hot alcohol. Water was then added to dissolve the soap and the solution was extracted with ether and treated as already described for the previous saponification. The ether was then removed in a tared flask and the whole dried for 1 hour at 105° and re-weighed. In this way the weight of unsaponifiable matter in each rat was obtained. The cholesterol present in this unsaponifiable matter was then estimated by the method of Windaus [1910] in an aliquot part of a solution in light petroleum in order to economise digitonin. The fraction of the solution taken was always such that it contained, as far as possible, the same weight of unsaponifiable matter. After removal of the light petroleum the unsaponifiable matter was dissolved in 10 cc. 95 % ethyl alcohol and precipitated in boiling solution with 0·2 g. digitonin in 90 % alcohol. In this way the weight of cholesterol digitonide weighed was kept fairly constant. After standing over night the precipitate was collected in a Gooch crucible, washed first with ether and then with boiling water

until free from digitonin, dried for 1½ hours at 105° and weighed. From the weight of the digitonide, the total cholesterol in each animal can be readily calculated. The method is tedious, but ensures that all the cholesterol present in the animal is estimated. Owing to the large number of manipulative operations, great care is necessary; further, any error in the actual estimation of the cholesterol is greatly increased owing to the amount of unsaponifiable matter taken being only a fraction of the whole. The results obtained are taken as representing the amount of cholesterol present although the possible occurrence of other sterols precipitable by digitonin must be kept in sight.

Results.

1. *Complete artificial diet.*

	Wt. of rat (pairs) g.	Cholesterol mg.	Wt. of rat (pairs) g.	Cholesterol mg.	Wt. of rat g.	Cholesterol mg.
	11·0	16·4	11·8	20·6	11	25·6
	10·9	17·4	12·0	21·0	11	26·8
	11·3	17·2	12·1	20·3		
	9·6	14·8	13·2	22·9		
	10·4	17·8	13·0	20·4		
			12·9	21·6		
Mean	5·3	8·4	6·2	10·6	11	26·2

	Wt. of rat g.	Cholesterol mg.	Wt. of rat g.	Cholesterol mg.	Wt. of rat g.	Cholesterol mg.
	23·1	67·3	27·5	73	34·3	76
	20·8	57·5	24·0	69	34·1	71
	22·0	56·3	30·1	64	36·3	74
	21·5	58·3	27·0	86	34·8	73
	19·2	52·3	29·0	88	38·0	98
	21·2	50·3	30·3	78	38·0	103
	19·4	43·9			35·0	92
					35·0	87
					34·0	86
					33·2	98
					31·5	88
					32·5	92
					31·3	85
					38·0	71
					40·0	69
					37·6	71
Mean	21	55	28	76	35	83

	Wt. of rat g.	Cholesterol mg.	Wt. of rat g.	Cholesterol mg.	Wt. of rat g.	Cholesterol mg.	Wt. of rat g.	Cholesterol mg.
	68	134	83	141	109	238	137	262
	64	122	100	161	114	183	140	282
	65	110	100	167	110	179	139	262
	70	143	90	163	111	195	140	268
	59	142	90	194	110	197	155	288
	57	141	100	162	121	263	155	279
	63	131	94	165	122	210	152	299
					105	212	148	280
					113	187	146	278
					112	229		
					113	209		
Mean	64	132	94	165	113	208	146	278

2. *Cholesterol content of* 100 *g. rats receiving the cholesterol-free diet.*

	Weight when weaned and put on to diet g.	Final weight when used for estimation g.	Cholesterol mg.	Cholesterol mg. per 100 g. rat
	42	103	225	219
	42	102	237	232
	46	98	219	223
	47	103	225	218
	42	102	227	224
	47	107	230	214
	41	107	226	210
	41	102	230	225
	37	100	224	224
	37	97	215	222
	50	94	214	229
	37	98	213	217
Mean	42	101	224	221

3. *Cholesterol content of* 100 *g. rats receiving cholesterol-free diet plus* 4 *mg. cholesterol per rat per day.*

	Weight when weaned and put on to diet g.	Final weight when used for estimation g.	Cholesterol mg.	Cholesterol mg. per 100 g. rat
	47	113	227	214
	37	106	243	225
	54	108	241	213
	55	110	241	213
	48	108	246	228
	56	110	238	216
Mean	49	109	240	218

It is recognised that there are objections to taking averages of figures such as these but the variations are not as great as might be expected considering the biological nature of the experiment, that the animals used were from many litters and that the ages of animals of similar weight often varied. It would have been an advantage if the number of animals used for estimation had been greater, but this was limited by the difficulty of raising sufficient litters from the limited number of stock animals selected for breeding for this experiment, and also by the tedious nature of each estimation. However, from the average figures it is possible to construct the curve in Fig. 1 in which the abscissae give the weights of the rat in g. and the ordinates the cholesterol content in mg. From this curve it will be seen that the cholesterol content of the young rat at weaning time is about 100 mg. and this figure is taken as the approximate content of all the other animals put into the other groups.

It would appear from this curve that a linear relationship exists between cholesterol content and body weight; further, the curve may be divided into two parts, one from birth to a weight of about 35 g., and a second part which is less steep, and it would appear that the increase of cholesterol with increase of body weight is definitely more marked in the early stages. This is shown more clearly by the curve in Fig. 2 in which are plotted as abscissae the ages of the

animals and as ordinates the amount of cholesterol expressed as mg. per 100 g. body weight.

It will be observed that there is a rapid increase in the percentage cholesterol to an age of 3 weeks followed by a fall to a value slightly above that at birth. The figures from which this curve has been constructed have been obtained by the conversion of some of the figures in the previous table to cholesterol percentages and their rearrangement into groups of animals of the same age. This was not possible with all, as records of all the ages wer

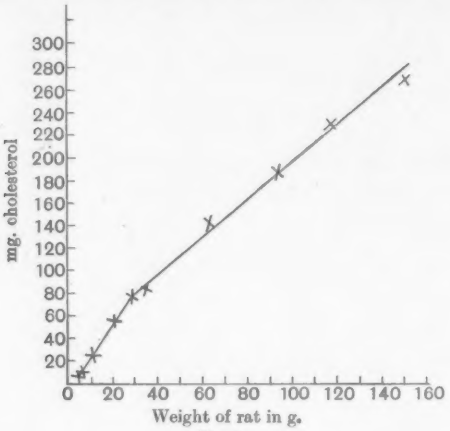

Fig. 1.

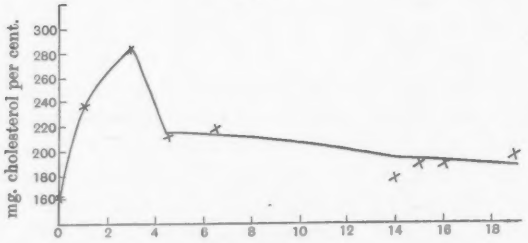

Fig. 2.

not kept. This curve is much less regular than the preceding one—a not unexpected result—but it would seem to indicate that the maximum cholesterol percentage corresponds approximately to the time of weaning at the age of 3 weeks, and this would suggest that it was due to completion of construction of nervous tissue. One other possibility which may explain this rise to a maximum is that the digestive tracts of the animals which were killed before weaning were not removed before estimation of the cholesterol content, and hence they may have retained in it some sterol from their mother's milk, but it is difficult to explain the results on this fact alone.

Cholesterol content of rats on the cholesterol-free diet at approximate weight of 100 *g.*

From an initial average weight of 42 g. to a final average weight of 101 g., the cholesterol has increased from 100 mg. (obtained from the curve in Fig. 1) to 224 mg. Using the figures reduced to 100 g. body weight, it is seen that the ratios of the final to initial body weights, and of the final to initial cholesterol contents are 100/42 and 221/100, *i.e.* 2·4 and 2·2 respectively; in other words, the rate of increase in cholesterol content has been very nearly as rapid as that of body weight. As the animals received no cholesterol over this period of 6 weeks during which their average weight rose from 42 to 100 g. and as their cholesterol content rose from 100 to 221 mg., an increase of 0·121 g. per animal, it would seem clear that cholesterol has been synthesised.

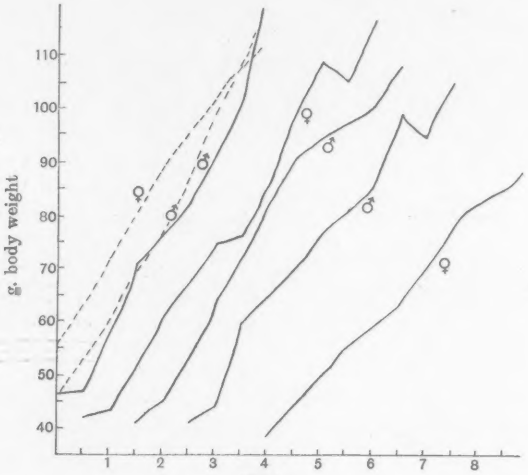

Fig. 3.

That the growth of the animals on the cholesterol-free diet was quite normal is shown in Fig. 3 in which are given the growth curves of six animals receiving this diet and taken at random. For comparison, there are given as dotted lines what are considered in this laboratory to be normal curves for animals receiving a diet complete in every respect.

Each animal received during the 6 weeks of diet III approximately 170 mg. of cholesterol, and had it all been stored, the total content of the animals which already had 100 mg. at the beginning, would have been 270 mg. even if synthesis had not occurred. It is curious that the average figure per 100 g. body weight in this group is 219 mg., whereas of those receiving no cholesterol (group 2) the average figure is 221 mg., the ingestion of 170 mg. having made no difference to the final cholesterol content of the animals of

group 3. It is to be observed that in group 1 (complete artificial diet) the cholesterol content of 100 g. rat is about 190 mg. whereas in both groups 2 and 3 the figure is about 220. No explanation of this difference has been obtained.

Sterol excretion.

Cholesterol was estimated in the ether extract of the dried faeces after 72 hours' extraction. The average figure per animal over a weekly period on the cholesterol-free diet was 0·0198 g. which would give for the 6 weeks of the experiment a total of 0·119 g. On diet III (poor in cholesterol) the excretion for the whole period was 0·126 g. per animal.

Thus the total sterol synthesised (that in the tissues was 0·121 g.) was 0·240 g. by each animal in 6 weeks.

SUMMARY.

1. A curve showing the relation between the body weight of rats from birth to 150 g. weight and the cholesterol content has been constructed for animals reared on the complete artificial diet used in this laboratory.

2. Rats fed from weaning time until they had reached 100 g. weight on a cholesterol-free diet showed an increase in cholesterol content from 100–220 mg.; the animal body would thus seem capable of synthesising cholesterol.

The writer wishes to thank Professor J. C. Drummond for his kindly help and suggestions, and the Government Grant Committee of the Royal Society for a grant from which part of the expenses of this research were defrayed.

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LXV. SOME PHOSPHORUS COMPOUNDS OF MILK.

I. THE PRESENCE IN MILK OF ORGANIC ACID-SOLUBLE PHOSPHORUS COMPOUNDS.

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Milk, as the sole foodstuff of the majority of young, growing mammals, contains all the substances necessary for this growth—proteins, fats, carbohydrates, salts and certain as yet chemically unknown factors. The necessity for further knowledge concerning these unknown substances is evident and urgent, and the present communication deals with the earlier stages of an investigation of the organic acid-soluble phosphorus compounds which are to be found in protein-free milk filtrates.

My attention was first drawn to these substances during the course of an enquiry into the nature of the acid-soluble phosphorus compounds of blood [Kay and Robison, 1924, 1, 2], when it was found that blood corpuscles contained at least two such esters, both hydrolysed by acids. One was hydrolysed by extracts of bone and of other tissues, whilst the other was more refractory towards enzyme activity. An average of 30 % of the organic acid-soluble phosphorus of blood was present in the more readily hydrolysed form. The view that these esters play an important part in bone formation has been supported by much evidence brought forward by Robison and his co-workers, and their possible rôle in carbohydrate metabolism [Kay and Robison, 1924, 2] and in the acute acidosis which follows ammonium chloride ingestion [Kay, 1924] has also received attention. The likelihood that young animals might receive their organic phosphorus direct from the milk, though, as experiments shortly to be described will indicate, a somewhat remote one, led me at the time to search for similar substances in milk. It was then found that phosphorus compounds, very similar to those occurring in blood corpuscles, were present in a free state in fresh milk in important quantities.

Although during the last 30 years a certain amount of intermittent attention has been paid to the organic phosphorus compounds of milk other than caseinogen, little is known as to the actual chemical state of the phosphorus, and still less has light been thrown on to its possible biochemical significance. It has been accepted for some time, in spite of sporadic denials with regard to

the last two forms, that phosphorus occurs in organic combination in milk in at least three forms—as caseinogen, as lecithin, and also in protein-free, lecithin-free milk filtrates. The work of Osborne and Wakeman [1915, 1916] on the phosphatides of milk has established their presence and identity firmly, one having been shown to be lecithin, another being found to be a diaminomonophosphatide. But we still do not know how the phosphate group is united to the rest of the molecule in caseinogen, nor whether there is any biological necessity for the presence of this group in the molecule of the protein. Our knowledge of the acid-soluble organic phosphorus compounds is, up to the present, hardly more than nominal. Apart from having given names which are somewhat misleading to these compounds the earlier workers can only be said to have rather shakily established their presence.

It is not surprising that discussion should have arisen as to the bare fact of the existence in milk of compounds belonging to the last group when one reviews the earlier methods used for getting rid of the other milk constituents. Siegfried [1896], for example, succeeded in separating a phosphorus-containing, non-protein substance from milk by precipitating the caseinogen with acetic acid and removing the albumin and globulin by boiling the filtrate. He then precipitated the free phosphates with calcium chloride and ammonia, and boiled the filtrate with excess of ferric chloride and ammonia. The precipitate formed contained iron, phosphorus and nitrogen, and was of roughly constant composition. It was variously called "nucleon," "carniferrin" or "phosphorfleischsaüre," the last two because it was considered to be allied in composition to a substance obtained from muscle, using a similar technique. Wittmaack [1897] went even further. He precipitated the caseinogen from human milk by warming the diluted milk to 50° with dilute hydrochloric acid, then boiled the filtrate to coagulate the albumin and globulin, and examined the protein-free filtrate for "nucleon." Sikes' [1906] criticism is not without justification, that this treatment hardly errs on the side of caution, and that any "phosphocarnic acid" found in the later stages might well be produced during this drastic process of protein precipitation. He instances Bayliss and Plimmer's [1905] finding that part of the caseinogen phosphorus may readily be split off in organic, non-protein form. Sikes asserted that no "phosphocarnic acid" was present in the milk he was investigating—human milk—when the protein was precipitated with tannic acid mixture in the cold, though he gave no experimental details to support his statement.

There is, however, little doubt that organic phosphorus compounds are present in the filtrates from cow's, goat's and human milk from which caseinogen and other proteins have been carefully removed, as the experiments outlined in this communication abundantly show. Methods of protein precipitation have been employed which are not open to the above criticism, and further, when more than one method has been applied to the same milk sample, the quantitative results have agreed well.

(It is of interest to note that "lecithin" in milk has passed through the

same stages of assertion [Nerking and Haensel, 1908], denial [Njegoram, 1913] and re-establishment [Osborne and Wakeman, 1915].)

Cow's, goat's and human milk have been investigated, and a readily hydrolysable organic phosphoric ester has been found present in all samples examined. For the normal milk of each species there is a fairly constant amount of this ester present, but the quantity differs from one species to another. It varies somewhat during the period of lactation, being greatest in goat's milk in the colostrum, and slowly diminishing in the later stages. (This aspect is still under investigation, and will not be dealt with here.) That these amounts are by no means negligible may be the more readily appreciated when it is pointed out that, in the case of human and of goat's milk, the readily hydrolysable phosphoric ester accounts on the average for between 10 % and 20 % of the total milk phosphorus, including that of caseinogen. In cow's milk the proportion of hydrolysable phosphorus is about 5 %.

Experimental.

In the earlier experiments fresh milk was obtained and kept as cool as possible until the proteins had been precipitated. The fact that the similar hydrolysable organic phosphorus of blood diminishes on standing, particularly if the blood be laked, owing to the activity of a phosphoric esterase in the corpuscles, suggested that a similar enzyme might also be present in milk, but it was found later that there was no appreciable diminution in the amount of readily hydrolysable phosphorus in milk after it had been allowed to stand for some hours at room temperature, or even in presence of antiseptic at 38°.

Methods of removal of proteins.

The first method used was to precipitate the caseinogen by bringing the diluted milk to the isoelectric point of this protein with dilute acetic acid, and then to throw down the albumin and globulin from the filtrate by adding sufficient trichloroacetic acid solution to make a final concentration of this acid of 2·5 %. The neutralised filtrate was used for the phosphorus determinations.

The second method, and the one usually employed in the later experiments, was to carry out the whole of the precipitation in one operation by the use of "dialysed iron." A perfectly clear, iron-free filtrate, giving no biuret reaction, and containing very nearly as much hydrolysable phosphoric ester as the filtrate prepared by the first method, could thus be obtained. The filtrate from the dialysed iron precipitation contained, however, much less free phosphate and was therefore more useful for determinations of increase of this ion. This diminution in free phosphate is the result of two causes: (1) caseinogen takes down with it the minimum of free phosphate if it is precipitated in the neighbourhood of its isoelectric point (p_H 4·7) [György, 1923], whilst the dialysed iron precipitate was well on the alkaline side of this, and (2) there was some formation of insoluble ferric phosphate.

Using the same sample of milk in each case and the phosphoric esterase

of bone as the hydrolytic agent the following results were obtained for the quantities of hydrolysable phosphoric ester present.

Table I. *Hydrolysable phosphorus in mg. per* 100 *cc. milk.*

	Proteins precipitated by acetic and trichloroacetic acids	Proteins precipitated by dialysed iron
Cow's milk	4·6	4·2
	4·7	4·9
	5·0	4·5
Average	4·77	4·53
Goat's milk	17·1	17·1
,, colostrum	25·4	23·0

From the above figures it appears possible that in addition to most of the inorganic, a small fraction of the hydrolysable organic phosphorus may have been carried down by the dialysed iron, but this, for present purposes, is insignificant.

In order to get complete precipitation of the proteins from milk with dialysed iron, certain precautions are advisable. It is better to use quantities of milk which are not too large, and the following volumes for normal goat's milk work well. 30 cc. milk are diluted to 120 cc. with distilled water. 45 cc. of 1·5 % dialysed iron (prepared by diluting B.P. *liquor ferri dialysati*) are run slowly from a separating funnel into the diluted milk, which is held preferably in a stout bottle to allow of continuous and fairly violent shaking. After the whole 45 cc. has been introduced, the bottle is shaken well and allowed to stand 10 minutes. The filtrate is usually perfectly clear. Rather less dialysed iron is required for 30 cc. cow's milk, usually from 35–40 cc. being sufficient[1].

The filtrate is now made faintly pink to phenolphthalein with a few drops of $N/10$ NaOH, and allowed to stand. If a precipitate appears it is filtered off (it does not contain organic phosphorus). The filtrate is now ready for the action of enzymes or other agents.

The method employed throughout for the determination of free phosphate was that of Briggs. A comparison between the results obtained by this method, and those obtained by the Neumann method as adopted for micro-estimations by Robison [1923] using the same milk filtrate, is given below. The hydrolysing agent used was a 5 % extract of rat bone. The figures represent mg. free phosphorus per 50 cc. of milk filtrate.

Table II.

	At 0 hours		After 18 hours at 38°		After 40 hours at 38°	
	Briggs	Neumann	Briggs	Neumann	Briggs	Neumann
A. Cow's milk filtrate + enzyme	0·34	0·33	0·67	0·64	0·75	0·70
B. Cow's milk filtrate + boiled enzyme	0·34	0·32	—	—	0·34	0·30
C. Cow's milk filtrate + water	0·24	0·23	—	—	0·25	0·25

The amount of enzyme used in this experiment was only half that used in the later determinations reported in this paper, in which the hydrolysis was completed, or very nearly so, in 18 hours.

[1] It has been found that the commercial "B.P." dialysed iron varies considerably in precipitating power and in acidity. The figures given above are therefore no more than an approximate guide.

The discrepancy, which is not large, between the values found by the two methods is doubtless due to the fact that a small amount of protein from the enzyme solution was present in A and B and interfered with the complete precipitation of the ammonium phosphomolybdate in the micro-Neumann method. Traces of this compound remained in colloidal solution and were thus not estimated. It is quite clear, however, from these figures that there is no analytical fallacy in applying the Briggs method to such milk filtrates.

Amounts of hydrolysable phosphoric ester present.

The amount of this ester present was determined by adding 5 % extract of rat bone to the neutralised milk filtrate in sufficient quantity to complete the hydrolysis in 18 hours, determining the free phosphate at zero hour and at intervals. It is convenient to use the bone enzyme, as some of its capabilities are known from previous work with blood filtrates, but, as will be seen, extract of intestinal mucosa, or of kidney will, under similar conditions, effect the same hydrolysis. In certain cases the last two extracts have been found to hydrolyse slightly more than bone extract, so that the figures for enzyme-hydrolysable ester when bone extract is used are to be taken as minimal.

Method.

10 cc. of the milk filtrate is measured into each of a series of test tubes, and 1·0 cc. 5 % bone extract and 0·25 cc. chloroform are added. Boiled enzyme replaces the active enzyme in the control tubes. The whole series, carefully stoppered, is then placed in the incubator at 38°. At intervals, two tubes are taken, and 2 cc. 15 % trichloroacetic acid are added to each, to precipitate the enzyme proteins. Of the filtrate from each tube, 10 cc. are taken for a Briggs' phosphate determination. The duplicates usually agree exactly. The results of a typical experiment are given below.

Table III. *Goat's milk, precipitated by dialysed iron.*

	mg. phosphorus per 100 cc. milk			
	0 hours	6 hours	18 hours	24 hours
Free P	7·9	24·1	24·5	24·5
Hydrolysed P	—	16·2	16·6	16·6

It will be observed that most of the hydrolysis takes place in the first 6 hours.

Table IV gives the collected results of a number of determinations using the milk of three different species.

It is clear that goat's milk contains considerably more phosphoric ester than either cow's milk or human milk, and in most of the experiments yet to be described, goat's milk has been used as the source of the ester. The relative constancy of the values shown above for cow's milk is probably due to the fact that each sample contained the mixed milk of several cows. The figure

for "non-hydrolysable" phosphorus is the difference between the total acid-soluble phosphorus in the milk filtrate (determined by the usual incineration method) and the sum of the inorganic and hydrolysable phosphorus.

Table IV. *Mg. organic acid-soluble phosphorus per* 100 *cc. milk.*

Cow's milk		Human milk		Goat's milk		Goat's colostrum
Hydrolysed by bone enzyme	Not hydrolysed	Hydrolysed	Not hydrolysed	Hydrolysed	Not hydrolysed	Hydrolysed
4·6	2·9	3·9*	2·0	16·6	—	25·4†
4·7	1·0	3·1*	2·0	17·1†	—	23·0†
5·0	3·6	2·5*	1·4	17·1†	—	
4·2	—	4·0	—	12·0	3·2	
4·9	—	2·4	2·0	8·6†	4·4	
2·5	2·1	3·1	4·0	8·9†	—	
4·5	1·1	[<0·5	—]	16·5	1·5	
4·4	3·5	[1·2	2·9]	12·4	1·7	
		[1·0	2·3]	10·6	—	
		4·0	1·9			
		3·1	4·0			
		3·2	2·8			
		3·3	3·9			
Av. 4·35	2·3	3·25	2·5	13·3	2·7	

* These samples late in lactation period from different mothers.
† Same sample, using different methods of precipitating proteins.

Note.—The three human milk samples in brackets are not included in the averages as they are probably abnormal. The first of the three was obtained from a mother whose child had just died from intestinal trouble, the last two were from hospital cases of difficult labour.

A comparison of these average amounts with those obtained for "nucleon" or "phosphocarnic acid" phosphorus by previous workers is of interest, although, as stated earlier, some of their methods would hardly be expected to give reliable figures. A short calculation from the published figures of Siegfried [1896], Wittmaack [1897] and Ellenberger, Seeliger and Klimmer [1902] has been made.

Table V.

mg. P per 100 cc. milk.

	Nucleon or Phosphorfleischsaüre P			Hydrolysable P (Present author)	Total organic acid-soluble P (Present author)
	Siegfried	Wittmaack	E. S. & K.		
Cow's milk	3·9	3·4	4·7	4·4	6·7
Goat's milk	—	8·7	—	13·3	16·0
Human milk	8·7	8·5	9·5	3·3	5·8

Only in the case of cow's milk is there approximate agreement. It would seem that there is some, but not a very direct or quantitative correspondence between the substances which have been called "nucleon" and the organic, phosphorus-containing substances present in milk filtrates, and estimated by the methods described in this paper.

The absence from milk of a phosphoric esterase.

Different samples of milk from animals of the same species, particularly goat's milk, gave markedly different figures for the hydrolysable phosphorus.

It was at first thought that this might be due to the presence of an enzyme in the milk itself.

No such enzyme appears to be present. Several experiments have been made (1) allowing the milk to stand for some hours at laboratory temperature, (2) allowing the milk to stand at 38° for 24 hours in presence of antiseptic, and in no case were more than minimal quantities of organic acid-soluble phosphorus hydrolysed. The results of two experiments are summarised in Tables VI and VII.

Table VI. *Effect of standing at room temperature.*

mg. P per 100 cc. milk.

	Free P	P hydrolysed by bone enzyme at 38°		Total acid-soluble P	Non-hydrolysed acid-soluble P
		After 6 hours	After 18 hours		
Milk drawn into cold bottle and precipitated at once	7·9	16·5	16·5	25·9	1·5
Same milk stood 4 hours at 20°	7·9	16·2	16·6	25·6	1·1
Same milk stood 8 hours at 20°	7·8	16·2	16·2	25·6	1·6
Stood overnight at 0°	8·8	16·2	16·4	26·8	1·6

Table VII. *Effect of standing at* 38°.

mg. P per 100 cc. milk.

	P hydrolysed by bone enzyme at 38°	
	After 6 hours	After 24 hours
Fresh milk precipitated at once	9·9	10·6
Same milk after standing 6 hours at 38°	9·4	10·1
Same milk after standing 24 hours at 38°	8·4	9·5
Same milk boiled for 1 minute	9·9	10·5
Boiled milk after standing 24 hours at 38°	8·8	9·9

If a phosphoric esterase is present at all in fresh milk it is there in no more than traces. Boiling for 1 minute appears not to affect the organic acid-soluble phosphorus of fresh milk.

The rate of hydrolysis of the acid-soluble phosphorus compounds by bone enzyme and by acid.

The rate of hydrolysis by bone enzyme was first determined in order to find out when hydrolysis could be taken as completed. Later, filtrates from the three kinds of milk examined were compared. The results, together with the curve given by a blood filtrate under similar conditions of hydrolysis [Kay and Robison, 1924, 1], are shown in Fig. 1.

In each case, using bone enzyme, well over 80 % of the hydrolysis occurs in the first 6 hours. Using double quantities of enzyme, the reaction goes to completion in this time so that determinations of the amount of hydrolysable phosphorus in a given sample of milk can be made in a single day.

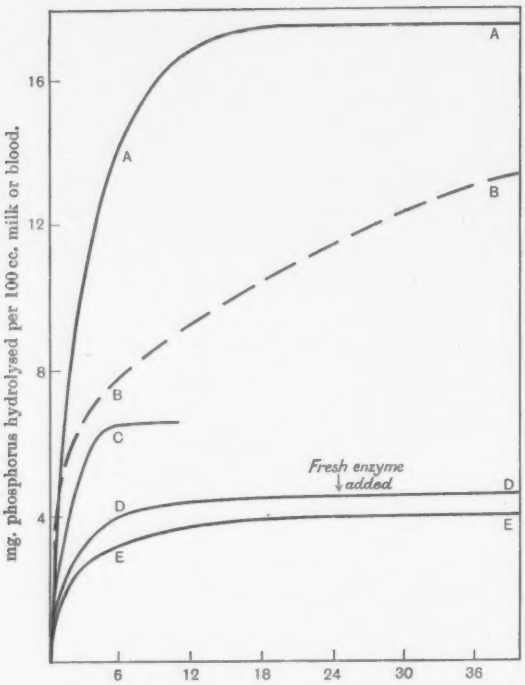

Fig. 1. A. Goat's milk filtrate + bone enzyme at 38°.
B. Goat's milk filtrate + $N/4$ sulphuric acid at 100°.
C. Rabbit's blood filtrate + bone enzyme at 38°.
D. Cow's milk filtrate + bone enzyme at 38°.
E. Human milk filtrate + bone enzyme at 38°.
Strength of enzyme: 1 cc. 5 % bone extract to each 20 cc. filtrate.

Action of various reagents on the phosphorus compounds.

The substrate is more stable to heat, acids and alkalies than its ready hydrolysis by bone enzyme would suggest. Results of experiments are summarised below.

Milk filtrate + antiseptic kept at 0° at p_H 8·2	Less than 5 % change in 3 months
Filtrate at p_H 8·2 boiled for 2 minutes	Less than 3 % hydrolysis
Prolonged boiling at p_H 8·2	Very slow hydrolysis
Autoclaving 1 hour at 120° (p_H 8·2)	11 % hydrolysis
$2N/3$ sulphuric acid at 38°	No hydrolysis in 18 hours
Autoclaving 1 hour with $N/10$ sulphuric acid at 120°	25 % hydrolysis
Boiling ½ hour $N/4$ sulphuric acid	22 % hydrolysis
Boiling 48 hours $N/4$ sulphuric acid	80 % hydrolysis
Heating with $2N$ sulphuric acid for 72 hours at 100°	Hydrolysis not yet complete
$N/2$ sodium hydroxide at 38°	Slight hydrolysis after 18 hours
$N/2$ sodium hydroxide at 100°	Marked hydrolysis in ½ hour and 80 % hydrolysis after 18 hours

The proportion of organic phosphorus which remains unhydrolysed after prolonged boiling with dilute sulphuric acid varies with the milk sample, and agrees in general with the findings in the bone enzyme hydrolyses, that a portion of the organic phosphorus, varying irregularly from 10–40 % of the total is not hydrolysable.

Plimmer [1913] found that glycerophosphates and similar esters containing PO_4 in the same grouping were quite stable to *N* alkali at 95°. The instability of the hydrolysable phosphoric ester, at least towards alkali, would indicate that it is not of the glycerophosphate type.

Action of digestive enzymes and tissue extracts.

In order to obtain information as to the possibility of the absorption of these milk compounds unchanged from the alimentary tract, a few experiments have been carried out on the hydrolytic power of digestive enzymes *in vitro* at 38°. At the same time it was thought worth while to investigate the distribution in various tissues of a phosphoric esterase similar to that present in bone. The results are summarised below.

Ptyalin. Very active ptyalin gave absolutely no hydrolysis, either at p_H 7 or at p_H 8·4 in 24 hours.

Pepsin. Armour's pepsin, very active towards fibrin, had no action whatever on the organic phosphorus of two milk filtrates at the optimum p_H for this enzyme.

Trypsin. Active trypsin (Liquor Trypsin Co., Allen and Hanbury), rapidly hydrolysing fibrin and caseinogen, was entirely without action on the organic phosphorus, either at p_H 7 or p_H 8·4.

Pancreatic juice, obtained by cannula direct from the duct of an anaesthetised dog, but not activated, had a slight but definite hydrolysing action. The same juice, activated by adding a 10 % extract of duodenal mucosa, hydrolysed as much organic phosphorus as did the bone enzyme. The effect was traced to the intestinal extract itself, which was able to hydrolyse the same, or even a slightly greater amount of the organic phosphorus than the bone enzyme.

Extract of pancreas and of spleen. A 5 % pancreatic extract from an adult dog gave in 18 hours almost as much hydrolysis (7·8 mg. P per 100 cc. milk) at p_H 8·4 as did bone enzyme (9·0 mg. P), whilst 5 % spleen extract was almost inactive (0·9 mg. P).

Intestinal extract. A 5 % extract of duodenal mucosa gave the same amount of hydrolysis as standard bone extract:

Table VIII.

mg. P per 100 cc. milk. Initial p_H 8·4. Time 18 hours.

Experiment	Hydrolysed duodenal extract	Hydrolysed bone extract
A	16·4	16·5
B	16·6	16·6
C	12·6	12·0
D	9·0	8·8

Intestinal juice. The juice was obtained by drawing the contents of the small intestine of a starving dog through cotton wool. The clear fluid so obtained was diluted to give a 5 % solution. At p_H 8·4 the diluted juice of two adult dogs gave in each case the same amount of hydrolysis as with bone enzyme. A fortnight-old puppy's diluted juice gave 28 %, a 7 days old puppy's 66 % of the amount hydrolysed by bone enzyme in the same time.

Gastric mucosa. A 5 % extract of the gastric mucosa of a dog brought about, at p_H 8·4, 75 % of the hydrolysis effected by bone enzyme. It was thus somewhat less active than intestinal mucosa.

Kidney extract. 5 % extract was slightly more active than bone enzyme at p_H 8·2, hydrolysing, in one experiment, as much as 16 % more organic phosphorus.

Mammary gland. A 10 % extract of the mammary gland of a lactating goat hydrolysed at p_H 8·4, 10·1 mg. P per 100 cc. milk in 24 hours, whilst standard bone enzyme in the same time hydrolysed 12·9 mg. P. The same mammary extract, when added to a protein-free filtrate from rabbit's blood, hydrolysed 5·9 mg. P %, whilst bone enzyme in the same time hydrolysed 9·2 mg. P %.

Blood. Diluted blood (one part blood to four of water) gave in 48 hours a small but definite amount of hydrolysis.

Muscle extracts. Fresh rabbit muscle extract, made by grinding up the tissue with sand and 10 times its weight of chloroform water and filtering, brought about, at p_H 8·4, 15 % hydrolysis of the organic phosphorus after 11 hours, and 22 % hydrolysis after 48 hours. By this time the hydrolysis had virtually ceased.

If the hydrolysable phosphorus compounds had been esters of the hexose-diphosphate type, this concentration of muscle enzyme and substrate should have resulted in hydrolysis of the order of 52 % in 11 hours, and 82 % in 48 hours [Kay and Robison, 1924, 2, Tables I and II]. Thus there is indirect evidence that these compounds are probably not to any large extent esters of the yeast hexosediphosphate type. As in blood, so in milk there would appear to be at least three forms of organic acid-soluble phosphorus present, the portion hydrolysable by bone enzyme in each case containing more than one substance. Consideration of the rate of hydrolysis of the organic phosphorus of milk filtrate by boiling with $N/4$ sulphuric acid leads to a similar view, the velocity constant falling off rapidly, and then becoming constant at about 8 hours, remaining constant until the experiment is discontinued at 48 hours.

Collecting the foregoing results into one table:

Table IX.

	Activity		Activity
Ptyalin	0	Intestinal extract	+ + + +
Pepsin	0	Spleen extract	+
Trypsin	0	Kidney	+ + + +
Pancreatic juice	+	Muscle	+ +
Intestinal juice	+ + +	Mammary gland	+ + +
Gastric mucosa	+ + +	Blood	+
Pancreas extract	+ + +	Bone extract	+ + + +

The effect of dialysis.

Raudnitz [1903], in criticising some of the "nucleon" findings mentioned earlier in the present paper, says that so long as it cannot be found in milk dialysates, the pre-existence of nucleon in milk is doubtful. The acid-soluble phosphorus compounds of milk may readily be separated from the milk colloids by dialysis at low temperatures in collodion sacs, and there would appear to be no reason to believe that they are not present in the same form in fresh milk. The results of a dialysis experiment are given below.

Table X.

mg. P per 100 cc. fresh goat's milk. Dialysis at 0°.

	In filtrate after pptn. of orig. milk with dialysed iron	Dialysate	
		6 hours	22 hours
Phosphate P	2·5*	11·4	12·8
Total organic acid-soluble P	13·0	11·2	12·0
Hydrolysable P	8·6	5·1	6·1
Non-hydrolysable P	4·4*	6·1	5·9

* The dialysed iron taken down much of the phosphate P and probably also some of the non-hydrolysable P.

It is clear that both types of organic phosphorus may be separated from goat's milk by dialysis. The experiment has been repeated using cow's milk, and the same result obtained.

Discussion.

The presence of acid-soluble, organic phosphorus compounds in milk having been established, questions arise as to the origin and function of these compounds.

They must originate either (1) by diffusion from the red blood corpuscle in which very similar compounds are present, or (2) by synthesis in the mammary gland, or (3) by breakdown of the cells of the mammary gland with discharge of their nuclear materials and other contents into the milk, or (4) by the breakdown of some more complex organic compound after secretion of the milk. The last possibility seems to be excluded by experiments mentioned early in this paper, the third is open to the objection that since, at least in goat's and in human milk, the amount of the phosphorus of the acid-soluble compounds is a relatively large fraction (15–25 %) of the total phosphorus of the milk, their derivation from this source would indicate a far more rapid breakdown of glandular tissue than appears possible.

That these phosphorus compounds may leak from the blood into the milk, as for example, urea and creatine [Denis, Talbot and Minot, 1919] appear to do, seems at first unlikely, since the phosphoric esters of blood are carried *in vivo* almost entirely in the corpuscles. It is possible, however, that when the corpuscles are slowly squeezing through the smaller capillaries, and are in intimate contact over a large portion of their surface with the capillary wall, conditions may exist for a direct interchange of substances between them and

the cells of the capillaries[1]. One would at least expect fundamental alteration in the surface relationships. As against the possibility of such diffusion from the blood is the fact that the bone-enzyme-hydrolysable phosphorus compound is preponderant in milk, whereas in blood it forms only one-third, or thereabouts, of the total phosphoric esters. This is, perhaps, not a very serious objection, since all recent work in this field goes to show that the phosphorus compounds in the tissues have a far more intimate kinetic relationship with one another than was previously realised, cf. the findings of Lawaczeck [1924] and of Hansman, Martland and Robison [1924] working on the dynamics of phosphoric acid compounds in blood.

The remaining possibility, that the substance may be definitely manufactured and secreted by the cells of the mammary gland derives some little support from the fact that an enzyme is present in mammary glands capable, *in vitro*, of hydrolysis of part of the acid-soluble phosphorus compounds, and possibly associated, *in vivo*, with their synthesis.

As regards the further history and possible function of these compounds, from the experiments with digestive enzymes and extracts of alimentary mucosa just described, it would seem on the whole unlikely that the organic phosphorus compounds are absorbed unchanged from the milk. Nevertheless, they are not attacked by the digestive enzymes before their arrival in the intestine, and in view of their relatively rapid diffusibility, there is at least the possibility of their being absorbed unchanged.

Table XI.

Forms in which phosphorus occurs in milk.

1. Free phosphate.
2. Phosphate loosely associated with caseinogen and not diffusible [György, 1923].
3. Caseinogen phosphorus.
4. Lecithin.
5. Diaminomonophosphatide [Osborne and Wakeman, 1915, 1916].
6. Acid-soluble phosphoric ester, hydrolysed by muscle phosphatase.
7. Acid-soluble phosphoric ester hydrolysed by bone enzyme, etc.
8. Acid-soluble organic phosphorus compound unaffected by these enzymes and stable to acids.

To the present author it would appear not unlikely that the imperative necessity for meeting the phosphorus requirements of the growing suckling has led, in the mammary gland, to the conscription of every possible synthetic mechanism by which the phosphorus content of the milk can be increased. Moreover, assuming that complete hydrolysis actually does precede absorption, and remembering that phosphorus compounds occur in milk in at least seven other forms of combination beside that of caseinogen (see Table XI), there is the supplementary possibility that this series has been evolved in relation to

[1] This conception may perhaps also be extended to the hypothesis advanced by Robison and his co-workers, that the organic esters in the red corpuscles play an important part in bone formation. A serious difficulty of this hypothesis has been the fact that at most only very small amounts of these esters are present, under normal conditions, in the plasma.

certain solubility and absorptive difficulties in the intestine. Having differing hydrolysability and therefore, during digestion, maintaining for a longer or shorter period their un-ionised PO_4, these compounds may be absorbed, with or without previous hydrolysis, in different forms, and at different levels in the small intestine, so that both calcium and phosphorus, each essential to the growing suckling, may be taken up most advantageously. This could be achieved if the concentration of PO_4''' ions were kept continuously low, so that the solubility product of $Ca_3(PO_4)_2$ should not be exceeded, and as little transformation as possible into this almost insoluble salt—and therefore the loss of both its irreplaceable constituents in the faeces—ensue.

The chemical identity of these substances is now being investigated, and from preliminary experiments it appears that one of the organic acid-soluble phosphorus compounds present in goat's milk filtrate, and hydrolysable by bone enzyme, is also capable of reducing alkaline copper solutions.

Summary.

It has been found that at least two acid-soluble compounds containing phosphorus in organic combination are present in cow's, goat's and human milk, one compound being readily hydrolysable by extracts of various tissues and by dilute acids, and one being considerably more stable.

The more readily hydrolysable compound is present to the average extent of 4·4 mg. P per 100 cc. in cow's milk, 13·3 mg. in goat's milk, and 3·3 mg. in human milk. In the last two milks, the acid-soluble organic phosphorus compounds account for from 10–25 % of the total phosphorus of the milk.

Both the hydrolysable and the stable phosphorus compounds pass readily through a collodion dialysing membrane which holds back protein but allows methylene blue to diffuse slowly.

Though not acted on by pepsin or trypsin, and only slowly attacked by pancreatic juice, the hydrolysable ester is probably broken down by the intestinal juice before absorption. Extracts of many tissues are also able to hydrolyse this phosphoric ester.

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LXVI. NOTE ON THE PHOSPHORUS CONTENT OF THE BLOOD OF RUMINANTS.

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(*Received April 3rd, 1925.*)

From the foregoing communication it will be seen that goat's milk contains a relatively large proportion of readily hydrolysable phosphoric ester. The experiments recorded below were begun in order to find out whether the phosphorus distribution in goat's blood could be in any way correlated with the unusual milk phosphorus distribution.

Abderhalden [1897, 1898] showed that goat's blood, in common with that of the other ruminants he examined (cow and sheep), contained much less total phosphorus than did the blood of the horse, the pig, the rabbit, the dog and the cat. His figures, collected from both these papers and recalculated in terms of milligrams P per 100 cc. blood, are as follows:

Table I.

	Sheep's blood		Ox blood	Bull's blood	Goat's blood	Pig's blood
	I	II				
Inorganic P	8·3	6·3	7·5	7·6	6·0	32·7
Total P	18·0	17·1	17·6	17·1	17·3	43·9
Lecithin P	8·9	9·6	9·4	8·8	9·8	9·2
Organic acid-soluble P	0·8	1·2	0·7	0·7	1·5	2·0
Acid-soluble P (Total – lecithin)	9·1	7·5	8·2	8·3	7·5	34·7

	Rabbit's blood	Dog's blood		Cat's blood	Horse's blood	
		I	II		I	II
Inorganic P	29·9	25·1	25·4	23·8	35·2	35·2
Total P	43·0	35·3	35·5	36·2	48·9	49·3
Lecithin P	11·3	8·2	8·0	9·3	11·6	11·9
Organic acid-soluble P	1·8	2·0	2·1	3·1	2·1	2·2
Acid-soluble P (Total – lecithin)	31·7	27·1	27·5	26·9	37·3	37·4

These figures show clearly the quantitative difference in the *total* phosphorus between the various animals, but at the time the analyses were performed it was not realised how easily autolysable was the organic phosphorus of the blood, and in consequence all the above inorganic phosphorus figures are much too high, and the organic phosphorus figures much too low.

It seemed of interest to determine whether the paucity of acid-soluble phosphorus in the blood of ruminants was due to lack of the "hydrolysable" or the "non-hydrolysable" organic phosphorus compounds reported by Kay

and Robison [1924] as being present in the blood of rats, rabbits, and man. If, for example, that fraction of the organic phosphorus which is hydrolysed by bone enzyme were completely absent from the blood of ruminants, the view that the organic phosphorus compounds of the hexosephosphoric ester type in the blood are directly concerned with ossification would lose some of its cogency. Actually, however, the main shortage of phosphorus, as compared with the blood of rodents or of man, is in the fraction that is *not* hydrolysable by bone enzyme.

Blood was taken in the slaughterhouse direct into oxalate, and was brought at once to the laboratory and the proteins precipitated in the usual way. Inorganic phosphorus was determined by the Briggs method; total acid-soluble phosphorus by ignition with a sulphuric-nitric acid mixture followed by the same colorimetric procedure; lecithin phosphorus by the method of Randles and Knudson [1922] and the hydrolysable phosphorus by Kay and Robison's method [1924, p. 757] with slight modifications.

The results are shown below. Average figures for the phosphorus distribution in human, rabbit's and rat's blood are appended for comparison.

Table II.

	P in mg. per 100 cc. whole blood						% red corpuscles haemato-crit	P in mg. per 100 cc. red corpuscles	
Animal	Total acid-soluble	In-organic	Organic	Hydro-lysable	Non-hy-drolysable	Lecithin P		Hydro-lysable	Non-hy-drolysable
Goat 1*	9·8	6·9	2·9	2·2	0·7	—	30	7·3	2·3
„ 2	7·6	4·0	3·6	2·5	1·1	7·5	30·5	8·1	3·6
„ 3	9·8	5·9	3·9	2·9	1·0	—	40	7·2	2·5
„ 4*	9·2	5·4	3·8	3·5	0·3	—	—	—	0·9§
„ 5	9·5	6·1	3·4	3·1	0·3	8·8	34	9·1	0·9
„ 6	9·2	5·9	3·3	3·0	0·3	—	35	8·6	0·9
Av. for 6 goats	9·2	5·7	3·5	2·9	0·6	—	34	8·1	1·8
Sheep 1	10·9	5·9	5·0	3·0	2·0	10·7	40	7·5	5·0
„ 2	11·4	5·7	5·7	3·6	2·1	9·9	39	9·2	5·4
„ 3	12·1	6·9	5·2	3·0	2·2	9·7	38·5	7·8	5·7
Av. for 3 sheep	11·5	6·2	5·3	3·2	2·1	10·1	39	8·2	5·4
Bullock 1	10·0	5·6	4·4	2·5	1·9	7·4	44	5·7	4·3
Cow	12·4	7·2	5·3	2·6	2·6	10·9	43	6·0	6·0
Bullock 2	9·4	4·9	4·5	3·0	1·5	10·9	49	6·1	3·1
Av. for 3 cattle	10·6	5·9	4·7	2·7	2·0	9·7	45	5·9	4·4
Man†. Av. for 5 men	25·8	3·1	22·7	6·4	16·3	11·8	43	14·9	38·0
Rabbit†. Av. for 10 adults	34·5	5·7	28·8	8·7	20·1	10·8‡	33	26·4	61·0
Rat†. Av. for 5 groups of adults	31·5	5·4	26·1	6·2	19·9	—	—	—	—

* Lactating.
† Taken from Kay and Robison [1924, Table I] and from hitherto unpublished figures.
‡ Average of 7 adults.
§ Assuming the average value of 34 % red corpuscles.

Table II indicates even more definitely than Table I the difference between the phosphorus partition in the blood of ruminants and in that of the three other species for which reliable figures can be given. Goats, in particular,

have practically no non-hydrolysable organic phosphorus, either in whole blood or in the corpuscles, whilst sheep and cattle have only small amounts. The quantity of phosphoric ester hydrolysed by bone enzyme is low for all three ruminants, being about one-half of that in the blood of the other species. What the metabolic significance of these extraordinary differences between the blood of ruminants and of other mammals may be awaits further experiment, but as far as classification is concerned it would appear from the evidence at present available that the unusual blood-phosphorus content is characteristic of the tribe *Ruminantia* only, and not of the whole of the sub-order *Artiodactyla*.

If the organic acid-soluble phosphorus compounds in goat's milk (average 16·0 mg. P per 100 cc. milk) come from the similar compounds in the blood of these animals (average 3·5 mg. per 100 cc. blood), there must be active concentration of this phosphorus going on in the mammary gland. The more likely hypothesis, in view of these figures, is that the milk compounds, at least to a large extent, are synthesised in the mammary gland.

With the micro-methods now available, the determination of the P/N ratio in blood or in extracted blood-stains affords a simple chemical test which might be of use in medico-legal investigations for distinguishing the blood of sheep, cattle and goats from human blood.

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LXVII. THE INFLUENCE OF WASHING UPON THE REPRODUCTIVE RATE OF *COLPIDIUM COLPODA*.

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(*Received April 4th, 1925.*)

In a recent paper Robertson [1924, 1] reports that he has observed allelocatalysis in cultures of *Colpidium* sp. both in hay infusions and in synthetic medium. He also explains our earlier failure to obtain this effect in cultures of *Colpidium colpoda* [Cutler and Crump, 1923] as being due to too great a concentration of the parent culture medium in the sub-cultures (a dilution of from 1/37–1/980), and claims that if the organisms had been washed until the dilution was considerably greater (1/9000 in his own case) the allelocatalytic effect would have been observed.

This is of course a direct contraversion of certain facts published by this author in 1923 [Robertson, 1923, p. 97], which demonstrated that the allelocatalytic effect occurs in sub-cultures in which large quantities of "autocatalyst" derived from the parent must have been present. "Into a drop, measuring about 0·08 cc. of freshly prepared hay infusion was isolated an infusorian (*Enchelys*) derived from a densely inhabited parent-culture which was 7 days old. At the same time two separately isolated individuals from the same parent-culture were introduced together into a similar drop of infusion. The following results were obtained:"

Culture number	Number of individuals initially introduced	Number of individuals	
		After 24 hours	After 48 hours
310 A	1	2	16
311 A	2	3	120

Here, if we may take it that the individuals were isolated into drops of a similar size to those used in other experiments, namely, 0·002–0·003 cc., the dilution is only 1 in 40, and moreover the parent culture had been the scene of very active reproduction.

Under similar conditions we have demonstrated that there is no trace of mutual acceleration in individuals of *Colpidium colpoda*, as is shown in Table I, compiled from the data given in a previous paper [Cutler and Crump, 1923]. The cells in question were isolated from a 2 days old culture in which there had been rapid reproduction and they were grown, in the usual way, in chambers containing synthetic medium.

Table I. *Reproductive rate after* 48 *hours in cultures in which the dilution of the parent culture liquid in the sub-culture is less than* 1 *in* 200.

	Reproductive rates	Average
One animal	2·0, 2·0, 5·1, 3·8	3·2
Two animals	3·6, 1·0, 2·4, 3·5	2·6

The present paper gives the results of a series of experiments on *Colpidium colpoda* in which the organisms were washed before isolation thereby reducing the concentration of autocatalyst carried over from the parent culture to an amount comparable with that in Robertson's experiments. The washing was effected by centrifuging a culture of known ancestry, *i.e.* age of culture and amount of reproduction which had occurred, siphoning off the supernatant liquid, and adding fresh medium to the remaining 1 cc., and repeating this process until a suitable dilution was reached. From the final liquid the animals were isolated into a capillary tube with about 0·2 mm.^{3} of fluid, and inoculated into a chamber containing a quantity of medium varying from 1·6–9·2 mm.^{3}, in which bacteria from a 1 day old agar slope had been emulsified. The dilution of the parent culture varies in these experiments from 1 in 2540 to 1 in 22,900.

It is necessary to point out that where a small quantity of liquid containing rapidly moving organisms is delivered from a fine capillary tube it does not follow that the same number of organisms will always be contained in every drop, hence one or two organisms from the same parent culture can be obtained in drops whose sizes vary only within very narrow limits.

Table II shows the results obtained in sub-cultures from 1 and 2 animals which have been washed before inoculation.

Table II. *Reproductive rate after* 24 *hours' growth of washed individuals derived from young parent cultures* (24–48 *hours old*).

Number originally isolated	Degree of dilution of fluid from parent culture in liquid of sub-culture		
	1 in 1,000–5,000	1 in 5,000–10,000	1 in 10,000–20,000
1	4·0, 4·1, 3·3, 4·1, 4·7, 3·3, 3·0	4·8, 3·0, 3·0, 3·0, 4·0, 3·0, 4·0, 4·0, 4·1, 4·6, 4·0	3·5, 3·3, 4·2, 3·8, 4·0, 4·0, 4·0, 4·0, 4·6, 4·0, 4·0, 4·1, 4·2, 4·6
Average	3·8	3·8	4·2
2	3·9, 3·8, 4·2, 4·2, 3·3, 3·9, 2·4, 3·6	3·3, 2·5, 3·0, 4·0, 3·3, 2·0, 2·9, 4·0, 3·9	3·5, 3·9, 3·0, 4·3, 4·2, 3·9, 4·2, 3·7, 3·8, 3·9, 3·7, 3·2, 3·9
Average	3·7	3·2	3·8
After 48 hours' growth the averages for the same series of cultures are:			
One animal	7·0	6·6	7·4
Two animals	5·6	6·1	6·6

It is obvious from the figures that in *Colpidium colpoda* allelocatalysis does not occur under the conditions in which Robertson has detected it both in *Enchelys* and in the other species of ciliate which he doubtfully assigns to the genus Colpidium. His description and figures of the animal, however

[Robertson, 1924, 2], indicate that he was not dealing with any species of the genus *Colpidium*, but more probably with one of the *Colpoda* genus.

The fact that this series of experiments shows higher reproductive rates at 24 hours than those given in our earlier paper [Cutler and Crump, 1923, 2], *e.g.* 3·8 as against 2·4, is due to the fact that in this series the cultures were fed with an unidentified species of bacterium [Cutler and Crump, 1924] which gives uniformly better results than are obtained where the cultures are treated with *Sarcina* as was the case in the earlier experiments.

To support his contention Robertson has also re-grouped some of the figures given in our earlier paper [Cutler and Crump, 1924] on *Colpidium colpoda*, and from these derives further support for his theory that the reproductive rate is retarded by a high concentration of the substances introduced with the organisms from the parent culture, because retardation appears to occur in the cultures of smaller volume into which the individuals were introduced. Column 1 of Table III shows the figures quoted by Robertson; the other columns he omitted in his paper. The numbers given are, however, all averages of individual reproductive rates and statistical treatment shows that the variation between the individual reproductive rates in any one group is as great as it is between those of different groups; and that therefore the apparent rise in the average reproductive rate from the smaller to the larger drops is a matter of pure chance; in short, there is no significant difference between any of the numbers in the original table.

Table III.

Vol. of substance in cu. mm.	Average reproductive rate in first 24 hours			
	One animal	Two animals	Three animals	Four animals
0–1·5	1·98	2·49	2·39	2·31
1·5–3·0	2·27	2·29	2·36	2·46
3·0–6·0	2·86	3·75*	2·46	2·50
6·0–9·0	2·87	2·84	(No cases)	3·30*

* Only two cases.

A further difference between the results obtained by Robertson and ourselves lies in the effect of washing on the organisms. Robertson [1924, 2] records that in his *Colpidium* sp. abnormal forms were sometimes produced in cultures from washed individuals, but in the case of our organism abnormal animals have never been seen. He has further found that washing affects the division of his species in that the animals in cultures from unwashed individuals tend to divide into four, those from individuals that have been washed once into either two or four, and those from individuals washed two or three times into two only. Again in the case of *Colpidium colpoda* this difficulty in the study of reproductive rates does not occur since reproduction in this species is by binary fission only, and washing does not retard the rate of reproduction as is shown in Table IV.

Table IV. *Average reproductive rates in cultures from washed and unwashed individuals.*

Initial inoculum	Unwashed. Dilution of parent liquid less than 1 in 35	Washed. Dilution of parent liquid 1/5,000–1/10,000	Washed. Dilution of parent liquid 1/10,000–1/200
One animal (after 24 hours)	4·45	3·75	3·86
Two animals (after 24 hours)	3·10	3·55	3·67
One animal (after 48 hours)	7·35	6·67	7·52
Two animals (after 48 hours)	6·75	5·70	6·55

SUMMARY.

In *Colpidium colpoda* allelocatalysis has not been observed in cultures either from washed or unwashed individuals.

Washing the animals has no deleterious effect upon them nor does it retard the reproductive rate.

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LXVIII. THE CHEMICAL NATURE OF THE MEMBRANE OF POTATO CORK.

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Introduction.

In all the vascular plants a very important part is played by cell walls the physical properties of which have been altered by deposition of fatty substances in and upon them. As these bodies modify considerably the penetrability of the wall to water and aqueous solutions it is very essential to have information as to the nature of the fatty deposit, its continuity, permanence and physical properties. Such information is gradually being accumulated in this laboratory, but a much fuller knowledge is required of the chemical nature of the fatty bodies involved than is at present available and the present paper is a first contribution to that end in the case of cork.

In the shoot of the flowering plant there are two very different types of fat-impregnated membranes. From its earliest appearance the shoot is clothed with a cuticle, a fatty layer which is continuous over the whole surface of the shoot, as a deposit upon the outer walls of the outermost layer of cells. Frequently in older stems this layer is replaced in later life by a layer of cork which has quite a different origin. In this case a layer of cells, always lying below the actual epidermal cell, enters into a stage of active division and growth. As a result, a series of cells are cut off to the outside of this meristematic cell of which the oldest is the outermost (Fig. 1 A). Within each of these cells there now forms an inner layer which possesses fat-staining properties and which is called the suberin lamella. Similar fatty substances impregnate the thin wall medianly placed between two adjacent cells and known as the middle lamella, but the continuous suberin lamella within the cell is laid down simultaneously in all parts of the cell and undoubtedly is formed from the contents of the cell itself which have accumulated at the surface of the protoplast (Fig. 1).

Within this suberin lamella is present a layer of cellulose impregnated to some extent with fat but quite recognisable when all fatty bodies are removed. The fatty substances which are under investigation therefore in cork are not in a continuous sheet, as in the cuticle, but mainly concentrated within a series of isolated lamellae lining individual cells, the suberin lamellae, and also to some extent diffused through a carbohydrate basis of cellulose or of pectates in the middle lamellae.

A brief historical account of our chemical knowledge of suberin has been given by Priestley [1921]. The basis of the assumption that it is fatty in nature is its behaviour towards fat stains such as Sudan III and Scharlach R. On the other hand, neutral fat solvents only remove a small proportion of these substances [Gilson, 1890] of which only a small fraction has been shown to be glycerides of fatty acids [Kügler, 1884]. The rest of the material of the suberin lamella is removed by boiling in alcoholic potassium hydroxide [Van Wisselingh, 1886–1895], and this extract is usually regarded as consisting in the main of salts of various organic (suberogenic) acids [Gilson, 1890]. The relation of these acids to fatty acids is not clear, but, in view of the fact that they all lose water at higher temperatures with consequent changes such as a rise in melting point and loss of solubility in fat solvents, they would appear to be of the nature of hydroxy-fatty acids.

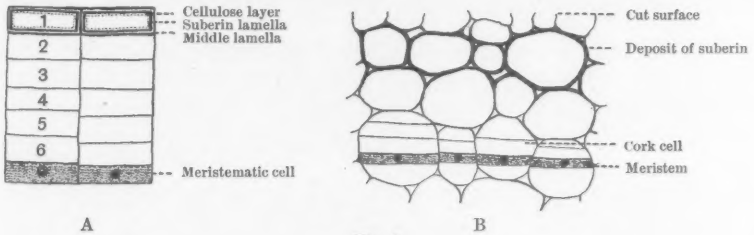

Fig. 1.

A. Series of cork cells formed from meristem, 1 oldest and outermost cell, 6 youngest. Details of wall shown in 1.

B. Formation of a new cork layer below cut surface of potato tuber.

In the present work, as a result of the experience gained with these somewhat intractable substances, the experimental methods of Gilson have been abandoned for methods similar to those used in the paint and varnish industries, where processes are involved in which linoxyn-like products are formed from the fats and oils derived from plants. It is hoped to show that such processes have considerable bearing upon the deposition of the suberin lamellae within the serried ranks of the cork cells.

Experimental Method.

The material chosen for examination was the skin of washed potatoes because it could be readily obtained in large amounts. It was washed in running water, dried fairly quickly some few feet from a fire in a well-ventilated room and then ground fairly finely.

The actual method of treatment, as now presented, is felt to be capable of yielding consistent semi-quantitative results for the various types of fatty substances involved and consists of three successive operations:

(1) Removal of chloroform-soluble material in a Soxhlet apparatus.

(2) Saponification of residual material with 3 % alcoholic sodium hydroxide.

(3) Treatment of alcoholic solution so obtained:
(*a*) Removal of unsaponifiable material.
(*b*) Removal of normal fatty acids.
(*c*) Removal of hydroxy-fatty acids.

(1) *Removal of chloroform-soluble material.*

The extraction apparatus used was made of metal and had a capacity of about 2 litres; it belongs to Messrs Rowntree & Co. of York, to whom grateful acknowledgments are due. This made it possible to free from fat, in one operation, quite a large bulk of tissue. The chloroform extract was distilled to small bulk, carefully washed into a tared dish, taken nearly to dryness on a water-bath and the last traces of liquid removed by repeated evacuation—while slightly warm—in a desiccator. With samples of the same tissue, results correct to less than 1 % can easily be obtained.

(2) *Saponification with alcoholic sodium hydroxide*[1].

The extracted tissue, after removal of chloroform by exposure to air was boiled for 6 hours with about 12 times its weight of alcoholic sodium hydroxide. The alcoholic liquid was filtered off and replaced by absolute alcohol in which the tissue was boiled for 1 hour before again filtering and adding a fresh supply of alcohol. This boiling with successive lots of alcohol was continued until the filtered extract ceased to show colour. The combined alcoholic extracts were taken to dryness on the water-bath.

The residual solid matter was quite free from suberin and dissolved completely in cellulose solvents.

(3 *a*) *Removal of unsaponifiable material.*

The mass obtained after removal of alcohol was taken up in water. Usually a large volume of water was required, as some of the materials present seem to have relatively insoluble sodium and potassium soaps. The aqueous solution was repeatedly extracted with ether[2] in a separating funnel until the ethereal layer was no longer coloured and a test sample gave no stain on a watch glass. The combined extracts were concentrated, carefully washed into a tared vessel and the remaining solvent removed in a vacuum desiccator. Evacuation was repeated until constant weight was reached. The practice of repeated evacuation was adopted in preference to the standard practice for unsaponifiable fractions, namely, heating to 110° for some hours, because the presence

[1] At first it was found impossible to obtain agreement between the amounts of material in subsequent stages 3 *a*, *b* and *c*. This was found to be due in large measure to the fact that the saponifying agent was made up from rectified spirit. When it was prepared by dissolving NaOH in its own volume of distilled water and making up to the required volume with absolute alcohol, the results for subsequent stages could be consistently repeated.

[2] The ether used was purified by re-distilling after standing over stick potash for several days. Unless so treated, consistent values could not be obtained, as the ethereal extracts were found to contain varying amounts of resinous bodies.

of relatively volatile bodies was early noted and it was thought that their preservation might be useful. The solution of sodium soaps was then made acid with dilute HCl, giving a suspension of free acids.

(3 *b* and *c*) *Removal of normal acids and "hydroxy"-acids.*

It was early noted that in this mixture of acids were bodies which, although soluble in ether, could not be removed by light petroleum (B.P. 40–60°) and were similar to those described by Lewkowitsch [1909] and Fahrion [1911] and called hydroxy-acids. The method for the separation of normal and hydroxy-fatty acids described by Lewkowitsch [1909] seemed to be the only one available and proved to be suitable. The suspension was repeatedly shaken with light petroleum (B.P. 40–60°) and the solvent layer separated until a test sample gave no stain on a watch glass. The combined petroleum extracts were treated in the same manner as for chloroform-soluble fat and the amount of normal acids thus obtained.

The aqueous suspension, freed from normal acids and consisting of hydroxy-acids, was filtered and the weight of hydroxy-acids determined after drying to constant weight in a vacuum desiccator.

The filtrate freed from fatty bodies was tested for glycerol.

Experimental.

Sharpe's Express potatoes were used in the following experiments. It was noted that, having removed the cork layer, leaving the potato in air for a few days resulted in the formation of a second, "regenerated," cork layer, and as this involved further suberisation and might provide useful data, these regenerated layers were also taken for examination each time within one week after removal.

With samples of the same cork layer semi-quantitative numbers could be obtained. The results of duplicate experiments are given in Table I.

Potatoes of gradually increasing age up to maturity were examined, the samples being taken from the same crop at different periods. Regenerated cork layers on these potatoes were also examined. Details are given below.

Normally formed Cork Layers.

Date of collection. 1924	Cork layer (code letter)	Details of potato
July 14	A	From potatoes of size slightly larger than a pea nut
	B	Larger than A. Maximum length about 1″
	C	Larger than B. Maximum length about 1¾″
August 26	D	Larger than C. Maximum length about 2″
	E	Larger than D. Maximum length about 3″
	F	Larger than E. Not quite mature
October 20	G	Larger than F. Mature

Regenerated Cork Layers.

Period of regeneration	Code letter of cork layer	Details of layer
Three weeks	H	Formed on A, B and C taken together
Three weeks	I	Formed on D
	J	Formed on E
	K	Formed on F
Four weeks	L	Formed on G

Table I.

	Weight of tissue taken g.	% on original dry weight of tissue			
		$CHCl_3$-soluble fat	unsap. material	normal acids	hydroxy-acids
Exp. 1	620	0·95	0·055	0·18	2·05
Exp. 2	663	0·95	0·058	0·17	2·17

Results of Examination of Skins A–L.

Table II. *Normally formed Cork Layers.*

	Cork layer	Dry weight % $CHCl_3$-soluble fat	Dry weight % unsap. material	Dry weight % normal acids	Dry weight % hydroxy-acids	Dry weight % normal plus hydroxy-acids
July 14	A	2·43	0·37	0·58	7·97	8·55
	B	2·74	1·86	3·12	6·28	9·40
	C	2·71	0·19	4·98	4·36	9·34
Aug. 26	D	3·30	1·53	3·23	11·23	14·46
	E	3·23	3·80	2·21	8·91	11·12
	F	3·41	4·83	5·93	6·61	12·54
Oct. 20	G	1·65	0·36	3·0	2·40	5·40

Table III. *Regenerated Cork Layers.*

Cork layer	Dry weight % $CHCl_3$-soluble fat	Dry weight % unsap. material	Dry weight % normal acids	Dry weight % hydroxyacids	Dry weight % normal plus hydroxy-acids
H	1·57	0·76	3·45	2·86	6·31
I	1·74	0·90	1·59	6·47	8·06
J	1·50	0·49	0·80	5·15	5·95
K	1·57	1·02	1·66	6·16	7·82
L	1·53	0·511	2·28	5·11	7·39

Examination of Products.

(1) *Chloroform-soluble fats.*

These bodies were all dark coloured, quite solid and possessed a rather sweet smell not unlike chocolate. The iodine numbers for the series A–L are given.

Table IV.

Normal cork layer		Regenerated cork	
Code letter	Iodine no.	Code letter	Iodine no.
B	70	H	74·6
C	60	I	76·0
D	68	J	74·0
F	64	K	63·0
G	56·7	L	62·4

The iodine numbers for other normal mature skins not included above, were all in the region 60–66, whilst for other regenerated cork extractions they varied from 70 to 75. Such tissues, if left in air for some weeks before extraction, yielded fats whose iodine numbers were considerably lower. A regenerated cork layer yielded a fat with an iodine number of 71, whilst after extraction 23 weeks later the iodine number was 53.

Fats from mature corky tissues had saponification values of 186–190 and 0·2–0·5 % ash in which sodium and potassium were detected. No marked solubility change was produced by exposure to sunlight for 40 days or powerful electric light for 70 days.

The amounts of constituent types of fatty acids from the fats of normal and regenerated cork were compared by saponifying them with 3 % alcoholic sodium hydroxide and following out the treatment as if for a skin.

	% unsap. material	% normal acids	% hydroxy-acids	% total acids
Fat from mature normal cork	15·3	50·0	33·6	83·6
Fat from regenerated cork	18·0	38·9	24·6	63·5

The aqueous residues after saponification were tested for glycerol, using the acrolein test. The fat from mature cork apparently contained only a trace, that from the regenerated cork evidently much more.

The normal acids thus obtained reduced alkaline permanganate slightly. They contained a little yellow colouring matter which was very difficult to remove. By repeated deposition from a mixture of light petroleum, ether and ethyl acetate, a pure white sample of an acid was obtained melting at 71–72°. Assuming mono-basicity, its molecular weight by the silver salt method was 283. The acid is assumed to be stearic acid. It had no iodine number.

The unsaponifiable material from both fats was heated to 70° on a hot plate for some hours. That from the young fat contained 23 % volatile material as against 0·2 % in the old fat. Tests of the non-volatile portions for phytosterol by heating with acetic anhydride according to usual practice were negative.

(3 *a*) *Unsaponifiable materials from cork.*

These bodies had invariably a strong penetrating odour and a yellow to brown colour. Attempts to detect phytosterol always failed. Heating to 70° removed in many cases considerable amounts of volatile material. Samples from regenerated cork contained in some cases as much as 70 % volatile bodies.

(3 *b*) *Normal acids.*

The invariable accompaniment of coloured extraneous matter, difficult to remove, has hampered progress. In order to remove it, it has been found best repeatedly to salt out the sodium salts with NaCl, finally acidifying and washing free from chloride. By this means a small amount of acid has been obtained which when moist is quite colourless but dries to a slate-grey colour. Its melting point is again 70–71°.

(3 *c*) *Hydroxy-acids.*

Although originally dark coloured, repeated salting out of the sodium salts gave a solid, quite white when moist, which again dried to a very pronounced slate colour, even *in vacuo* in the dark.

Gilson found great reduction of solubility with the suberogenic acids when heated in sealed tubes to high temperatures. Some hydroxy-acids from mature skins were heated in air for 27 days at 50°. They then showed a decrease in solubility of 50 %, indicating behaviour similar as regards solubility to the suberogenic acids. Gilson's procedure for obtaining the individual pure acids was followed without success.

Discussion.

In considering the data obtained it must be remembered that the fatty products obtained are derived not only from the suberin lamella itself but also from the fatty impregnations of other lamellae. Microscopic examination has revealed in normal cork layers the presence of an occasional epidermal cell outside the cork, indicating that a trace of the original cuticle may be present. In the case of regenerated cork layers, much of the extracted material will arise from the deposit—also termed suberin—which occurs in and along the wall of cells at the exposed surface (Fig. 1 B).

The chloroform extract of normally formed cork contains very little neutral fat. The bulk appears to consist of fatty acids with only traces of sodium and potassium soaps.

The values in Tables II and III are expressed as percentages of original dry weight of tissue. Obviously, comparisons must be drawn with caution, as on different occasions different amounts of extraneous tissue may be removed with the cork and be taken for examination. On these grounds such comparisons are not made in spite of what at first sight would appear to be interesting differences in chloroform extract percentages. If the total amounts of fatty acids released on saponification be compared with the amount of chloroform-soluble material, it will be observed that the ratio

$$\frac{\text{Hydroxy-acids + normal acids}}{\text{Chloroform-sol. bodies}}$$

is approximately constant. With the exception of D, in which the yield of acids on saponification is high, the ratio for *normal cork* layers only varies from 3·3–3·67 (Table V).

Table V.

Normally formed cork		Regenerated cork	
Tissue letter	Hydroxy-acids + normal acids / Chloroform-sol. fat	Tissue letter	Hydroxy-acids + normal acids / Chloroform-sol. fat
A	3·52	H	4·02
B	3·42	I	4·6
C	3·45	J	3·96
D	4·38	K	4·9
E	3·44	L	4·8
F	3·67		
G	3·3		

When the chloroform extract is small the acids released on saponification are low also and in proportion. The modifying factor may be the amount of extraneous tissue accompanying the pure cork layers. We may accept as a first conclusion that, in the normal cork layer at all stages of development, the proportion of substance soluble in fat solvent to fatty substances extractable after saponification is approximately constant. As the series contains tissues with increasing numbers of mature cork cells, the numbers increasing from 3 or 4 to 10 or 15 without any appreciable change in the above ratio, we may conclude that there remains in the suberin lamella (Fig. 1 A) a definite proportion of material soluble in fat solvents. This result is also indicated by other investigators working on bottle cork which consists of still older cork cells, but in which Kügler [1884] found some 9 % of substances soluble in chloroform (excluding cerin). Experiments with chloroform-extracted tissues show that the chloroform-soluble material is responsible almost entirely for the staining reactions of the suberin lamella with Sudan III.

In the regenerated cork layers a larger and variable proportion of the more resistant bodies is present (Table V). Here, however, it must be remembered that we are dealing not only with internal suberin lamellae but with an irregular though continuous layer formed in and on the cellulose walls near the exposed surface, from which one would expect an excess of resistant bodies, owing to a more complete "drying" of this more exposed layer.

It can be definitely stated that the chloroform-soluble material itself contains both normal and hydroxy-acids, and Kügler's statement that stearic acid is present in a chloroform extract of *Quercus suber* adds support to the tentative identification of stearic acid in the corresponding extract of potato cork. No fatty acids have been obtained which are liquid at normal temperature, and the solid nature of all fatty bodies isolated in this work, coupled with the fact that saponification values would agree with the presence of acids of the stearic type as major constituents, suggests that only small amounts of unsaturated acids are present. The iodine value may be due to these small amounts of unsaturated acids, together with any unsaturated colouring matter or unsaponifiable body, associated with the main bulk of saturated material in the extract. It will be seen that the saponification of such an extract of normal cork yielded some 99 % of products as non-saponifiable, normal and hydroxy-fatty acids, the normal acids being mainly stearic, and showing only slight reaction to alkaline permanganate. The unsaponifiable material present in the fats is characterised by its greater content of volatile bodies in the case of regenerated cork.

After the removal of the normal and hydroxy-fatty acids from the corky tissue microscopical examination showed the complete absence of fatty materials in the residues. The walls disappeared entirely under the action of cellulose solvents, a result obtained previously by Payen [1868] with potato cork. Payen, however, before extraction with alcoholic potash had soaked the tissue for days, first in 4 % HCl and then in 20 % acetic acid. Whilst, however, the

ratio of the sum of normal and hydroxy-acids to chloroform-soluble material remained constant, the amounts of each constituent were very variable (Tables II and III), this variability holding also in the regenerated cork layers. Coupling these facts, it is obvious that either the method of extraction is not reliable and the separation of hydroxy- and normal acids not efficient, or else there is genetic connection between the hydroxy- and normal acids, the one replacing the other and the whole remaining approximately constant. The consistent results in Table I negative the first alternative so that one is led to assume that the hydroxy-acids are formed at the expense of the normal acids. In the suberin lamella itself these hydroxy-acids are insoluble in fat solvents, and are only released on saponification, thus indicating some form of combination or condensation. Stearic acid is almost certainly present in the fat, and the normal acids from the products of tissue saponification seem to be very similar in all respects. The hydroxy-acids obtained by tissue saponification and by saponification of chloroform-extracted material behave similarly. These bodies plus the unsaponifiable material constitute all that is present in the walls of the cork cells. There can be little doubt that the suberin lamella owes some of its properties to the presence of normal and hydroxy-acids in a state of combination or condensation, and that the hydroxy-acids are derived from the normal acids by some process of oxidation, and if the bulk of these should consist of stearic acid, as is suspected, then perhaps saturated acids will have to be considered as a possible starting point.

Little can be said regarding the unsaponifiable material. It certainly contains no sterols. In the chloroform-extracted material glycerol is present in the normal cork in traces only but in appreciably larger amount in the regenerated cork. No glycerol has ever been detected in the alcoholic sodium hydroxide extracts. These facts are not in disagreement with the suggestion of Schmidt [1910] that the fatty acids first accumulate as glycerides, later being split up and the glycerol oxidised to CO_2 and water. The large amounts of volatile bodies present in the unsaponifiable material from regenerated cork whether from chloroform or alcoholic sodium hydroxide extracts may be by-products from the oxidative changes discussed. That these volatile bodies should be less in amount in older normal cork layers is to be expected. Dr P. W. Clutterbuck, working on the oxidation of stearic acid, has noted the appearance, as by-products, of bodies with similar appearance and properties.

Summary.

A method is described by which the fatty constituents of the cork layer of the potato skin may be entirely removed, leaving the walls completely soluble in cellulose solvents. The removed bodies can be separated into chloroform-soluble material, unsaponifiable material, normal and hydroxy-acids and semi-quantitatively estimated.

It is concluded that the suberin lamella of the cork cell arises by changes

taking place in the fatty material resulting in the appearance of bodies no longer soluble in fat solvents.

A relatively constant proportion of the soluble fatty substances never undergoes this change and this soluble component of the suberin lamella is mainly responsible for its staining properties.

In the main the suberin lamella consists of relatively insoluble normal and hydroxy-fatty acid complexes which can be released by prolonged saponification as soluble soaps.

The non-saponifiable substances of the regenerated cork layers contain considerable amounts of volatile bodies.

Glycerol is found only in the chloroform extracts and then only in traces except in the case of regenerated cork layers.

The bearing of these facts on the formation of the suberin lamellae in cork cells is discussed.

The author wishes to express his most sincere thanks to Professor J. H. Priestley for constant advice and help throughout the whole of the investigation, to Mr F. C. Steward, B.Sc., who assisted in carrying out many of the extractions, to the Royal Society for a grant for collection of material, and to the Department of Scientific and Industrial Research for a grant of maintenance.

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LXIX. STUDIES ON THE GROWTH OF YEAST.

I. THE INFLUENCE OF VOLUME OF CULTURE MEDIUM EMPLOYED.

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Report to the Medical Research Council.

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Ever since the controversy between Pasteur [1872] and Liebig [1870, 1871] as to the growth of yeast in a synthetic medium the problem has remained unsettled. It will be remembered that Pasteur claimed originally [1859] that he could obtain a good growth of yeast "dans un milieu formé uniquement de sucre, d'un sel d'ammoniaque et d'éléments minéraux." Although the work of Willaman and Olsen [1923] shows that it is highly probable that the cane sugar which Pasteur used was contaminated with growth-promoting substances, yet Fulmer, Nelson and their supporters at the present time claim that they can produce a normal growth of yeast on their Medium "F" which is composed of chemicals of the highest possible degree of purity. On the other hand, there are still a number of workers who claim that for the normal growth of yeast some substance of a "bios" nature is indispensable (Funk, Eddy, Williams *inter alios*). In fact, a crystalline substance with well-defined physical properties and marked "bios" activity has recently been described by Eddy, Kerr and Williams [1924]. [For further references and details of this controversy see *Med. Res. Council Report*, 1924, p. 54.]

Whatever may be the true solution to what we may call the "bios problem," the differences between the results obtained by Pasteur and Liebig have yet to be explained on an experimental basis. The following communication is an attempt to supply such an explanation in the hope that it may throw light on the "bios problem."

Liebig, in accord with his view that fermentation was a process which only occurs in non-living matter, emphatically opposed the idea of Pasteur that it was an expression of the living yeast cell. In support of his contention he stated that he had repeatedly attempted to obtain a growth of yeast under Pasteur's conditions without succeeding in doing so. In these attempts, as we shall see below, he did not inoculate as much yeast in his medium as Pasteur had done. The main difference therefore between the experimental conditions of these two observers lay in the different volumes of medium which

were employed for each yeast cell originally present. With this difference in mind, the experiments detailed in the following communication were undertaken. Their object was to determine the effects, if any, of alterations in the volume of culture medium used on the growth of single cells of yeast. The experiments fall into two classes, one in which the yeast was grown in a medium as free from "bios" as possible, the other in which various concentrations of "bios" extracts were added.

Owing to the difficulty of obtaining in any given experiment a large number of active single cell cultures, observations were also made on cultures containing two cells. The justification of the use of results obtained in two cell cultures as corroborative evidence of the behaviour of single cells will be given in the next communication (Part II).

Experimental.

General.

In view of the influences on growth which may occur owing to the presence of bacterial contamination (*vide infra*) the technique has been further modified to prevent contamination as far as possible and to determine with greater certainty those cases in which it may have occurred. With the exception of these modifications the general procedure was essentially the same as in the experiments previously described [Peskett, 1924]. The following media were used:

Medium 1. Basal medium + 50 g. per litre commercial cane sugar.
Medium 2. Basal medium + 50 g. per litre recrystallised cane sugar.
Medium 3. Medium 2 + 0·025 mg. per cc. of solid "bios complex."
Medium 4. Medium 2 + 1·0 cc. yeast extract per litre.
Medium 5. Medium 2 + 10·0 cc. yeast extract per litre.
Medium 6. Medium 2 + about 1·0 cc. "Kepler" malt extract per litre.

The basal medium, recrystallised cane sugar and "bios complex" were prepared as previously described. The yeast extract was prepared according to the method of Eddy, Kerr and Williams [1924] from fresh "D.C.L." yeast. After shaking with fuller's earth, the filtrate was made up to the volume of autolysate from which it had been prepared for use in these media.

It was also found to be more satisfactory to rinse the cover slips in glass-distilled water instead of acid alcohol before drying. The cultures were grown at 25°, and the yeast used was the same as in the previous experiments.

Preparation of drop-cultures.

Hanging-drop preparations were made by means of a silica pipette in the manner described previously. In view of the difficulty of removing vaseline from the cover slips before fixing and staining, this substance was only used to the extent of two small dabs placed at opposite corners of the depression

in the slide. This was quite sufficient to allow the cover slip to be picked up readily with the slide. When in position, the cover slip was sealed at its edges with warm paraffin wax.

Parent culture.

As in the previous experiments, great difficulty was experienced in obtaining a large number of drops containing single cells which would grow satisfactorily. The mortality among the drop-cultures seemed to vary to a certain extent according to the manner in which the parent culture from which they were derived was obtained. After many trials it was found most satisfactory to use a culture which had been grown at 25° in 2·0 cc. of Medium 3 for not less than 2 or more than 4 days; the best results being obtained when a small loopful of a culture at least 14 days old was used for inoculation. To avoid the risk of contamination by wild yeasts, the stock culture was occasionally started afresh by transferring a colony derived from a single cell in a drop preparation to a fresh tube of Medium 3.

Drop volumes.

By direct measurement with a high power objective and a calibrated fine adjustment of the microscope the depths of a large number of different-sized drops of Medium 2 were determined. At the same time their diameters were measured by means of an eyepiece micrometer. These drops had been dusted with lycopodium powder to enable their surfaces to be focussed. Since the shape of the drops was found to be approximately that of a segment of a sphere, the usual formula was applied, viz. $V = 1/3\pi\,(3r - h)\,h^2$. From this formula the volumes of these drops were calculated and plotted on a curve against their diameters.

In the following experiments only the drop diameters were measured, and from these the volumes were read off from the curve obtained above. It will be seen therefore that the values given for drop volumes, though recorded in cubic millimetres, are only empirical.

Observation and staining of the cultures.

In order to minimise errors of evaporation and condensation of the drops, which might occur owing to changes of temperature while the cultures were under observation, a warm stage (at 25°) was used.

Fixing and staining the cultures. The technique described previously involved a long period of drying before the cultures could be fixed. Since in many cases the cultures became contaminated during this process a considerable amount of time has been spent in trying to overcome this difficulty. The technique finally adopted was as follows:

At the conclusion of the period of observation the position and approximate size of the drop-cultures were marked on the upper surface of the cover slip by means of Indian ink. The cover slip was then removed from its slide and

the upper surface coated with a thin layer of paraffin wax. A small ring and an identification mark were then etched round each ink mark by means of hydrofluoric acid, the excess of acid being washed off carefully with a few drops of water. The cover slip was warmed to evaporate the greater part of the water from the drops, and whilst still warm any traces of paraffin wax or vaseline were removed with a rag moistened with xylol. The preparation was then immersed for a few seconds in a dilute solution of collodion[1], and transferred rapidly to 80 % alcohol. After a few minutes in that fluid it was transferred to 50 %, and thence to 10 % alcohol. The preparation was then stained and mounted as described in the previous experiments. The position and size of the cultures having been fairly accurately marked, it was only necessary to search these circumscribed areas for bacterial contamination.

It was found that, by using this method, only in a very few instances did any of the yeast cells of a given culture become detached from the preparation. Moreover, the final preparations were comparatively free from granular débris, even when viewed under the highest powers of the microscope, provided that the stains were filtered before use. For these reasons I have described the method at some length in the hope that it may be useful to other workers in this field.

Results.

Method of consideration.

In each experiment I have plotted on a graph the number of cells present at each observation in every culture which showed growth. All the cultures considered were found to be free from bacterial contamination according to the method described. Fig. 1 illustrates the results obtained in a typical experiment.

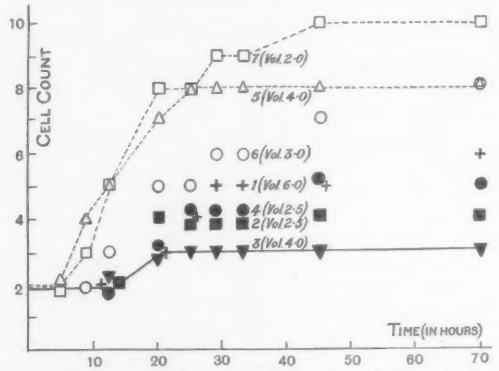

Fig. 1. Growth of two-cell cultures on synthetic medium.
Culture No. 3 (▼) showed the slowest growth.
Cultures No. 5 (△) and No. 7 (□) showed the quickest growth.

[1] Prepared by the addition of 5·0 cc. of collodion solution (containing 3 g. of pyroxylin in 75 cc. ether and 25 cc. alcohol) to a mixture of 25 cc. alcohol and 25 cc. ether.

In order to save space, the curves have been omitted and the results given in tabular form.

Arrangement of tables.

In one experiment the points plotted on the graph fell very close together, there being little variation in the growth recorded in the individual cultures. In this experiment (4 A) the drop volumes of all the cultures are given in the first column of the table, under "Average growth."

In other experiments (1 B, 6 B, 8 A) one culture in each experiment showed considerably quicker growth on the graph, and its drop volume has been recorded in the second column of the table, under "Quickest growth." The remaining cultures in each of these experiments, showing little variation in growth, are considered in the first column of the table.

In most of the experiments the graph obtained has been similar to that shown in Fig. 1. Here it will be observed that there is considerable variation in the growth recorded in the individual cultures. In these experiments the drop volumes of the cultures showing the quickest growth (*e.g.* nos. 5 and 7 in Fig. 1) have been recorded in the second column; whilst the volumes of those showing the slowest growth (*e.g.* no. 3 in Fig. 1) have been given in the third column of the table, under "Slowest growth." The volumes of the remaining cultures in these experiments are given in the first column.

To determine the rate of growth I have in all cases studied the growth curve from the last point before the first division occurred. Any acceleration of growth due to shortening of lag period alone has not been considered.

Growth on synthetic media.

Table I. *Drop volumes in mm.*^{3}.

	A. Single cells			B. Two cells		
Exp.	Average growth	Quickest growth	Slowest growth	Average growth	Quickest growth	Slowest growth
1	8, 58, 23, 5, 64, 90, 90, 43, 31, 14	58	10	13, 23, 64, 6, 43, 13, 14, 31	43	—
2	—	—	—	17, 10, 6, 13, 146	23	23
3	—	—	—	4, 13, 8, 5	13, 13	5
4	4, 13, 4, 13	—	—	6, 3, 2·5, 2·5	2, 4	4

In Table I the growth of 46 cultures has been considered, in volumes varying between 2 and 146 mm.^{3}. All of these were grown on "synthetic medium" (Medium 2) and found to be free from bacterial contamination so far as the technique allows it to be judged. In the range of volumes studied, there was little evidence of any constant influence of differences in volume on the growth. Thus, the evidence of acceleration in the larger volumes and retardation in the smaller shown in Exp. 3 B is counterbalanced by the uniform growth observed in the volumes shown in Exp. 4 A. In all the remaining experiments it will be seen that fairly uniform growth has been observed over a wide range of volumes; in every case in volumes larger than that in which

the quickest growth has been recorded and smaller than that in which the slowest growth occurred, and *vice versâ*.

In synthetic media, therefore, within the range of volumes studied, I cannot attribute any of the variations in growth which occurred to differences in the volume of culture medium employed.

Growth on media containing "bios" extracts.

Table II. *Drop volumes in mm.*^{3}.

		A. Single cells			B. Two cells		
Exp.	Medium	Average growth	Quickest growth	Slowest growth	Average growth	Quickest growth	Slowest growth
5	5	143, 238, 74, 90, 157, 362	90, 45	77	—	—	—
6	4	19, 26, 22, 23, 36, 48, 2, 10, 6, 13, 13, 6	115	48, 29	17, 27, 31, 17	107	—
7	6	7, 13, 9, 4	57	9, 43, 13	64, 14, 23, 48	10	1, 0·5
8	3	178, 161, 213, 260	106	—	235, 178, 178, 161, 107	270	23, 74

In Table II the growth of 57 cultures, on media containing various concentrations of growth-promoting substances, has been considered. These were all free from bacteria as far as could be judged. The volumes varied between 0·5 and 362 mm.^{3}.

In the first place, it will be seen that in five experiments (5 A, 6 A, 7 A, 7 B, 8 B) there were cultures which showed extremes of growth in both directions. In these experiments there were 16 such cultures. As regards 15 out of these 16 it is true to say that the cultures which showed quicker growth had larger volumes than the corresponding cultures which showed slower growth and *vice versâ*. In media containing "bios" there is, therefore, evidence that the quickest growth occurs in volumes larger than those in which the slowest growth is observed.

In order to simplify the consideration of all the experiments in the table I have drawn up the following analysis. Out of 18 cultures which showed exceptionally quick or slow growth 8 showed quick growth and 10 showed slow growth.

Of the eight showing quick growth the volumes of	Exp. nos.	Of the ten showing slow growth the volumes of	Exp. nos.
Four were largest	6 A, 7 A, 6 B, 8 B	Three were smallest	7 B, 8 B
Two were smallest	5 A, 8 A	None were largest	—
Two were intermediate	5 A, 7 B	Seven were intermediate	5 A, 6 A, 7 A, 8 B

In the 9 cultures in intermediate volumes there was no constant relationship between volume and growth. Of the remaining 9 cultures, 7 showed definite evidence of acceleration of growth in the largest volumes or of retardation in the smallest, and only 2 showed the opposite effect.

Secondly then, in view of the fairly uniform growth obtained in Exp. 5 A over a very wide range of volumes, and in consideration of the aberrant growth noted in the 9 cultures in intermediate volumes (considered in the analysis) it is difficult to ascribe any of the variations in growth which occurred to

differences in volume. But in those cases where extreme variations in growth occurred in association with extreme differences in volume, there was distinct evidence of acceleration of growth in the larger volumes and retardation in the smaller in the majority of cases (77 %).

Thirdly, in the media containing "bios" extracts I have not observed slowing of the growth in the largest volumes, and in only two cases out of eight did acceleration occur in the smallest volumes. Robertson [1923] claims that the growth of infusoria is approximately inversely proportional to the volume of medium in which they are grown. This is not true of the yeast used in these experiments.

Growth in presence of bacterial contamination.

Table III. *Exp.* 9. *Medium* 2. *Single and two-cell cultures.*

	After 72 hours at 25°	
Cultures considered	Grown	Not grown
16 sterile (control)	9	7
16 infected	1	15

In Table III I have recorded the results obtained in an isolated experiment in which some of the cultures were intentionally contaminated with bacteria. The infected cultures had been lightly inoculated with a pure culture of a short rod-like bacillus obtained from the laboratory air. In its cultural characteristics it resembled *B. fluorescens liquefaciens*. Although the growth recorded in this experiment was not good even in the sterile control cultures, there was a definite inhibition of growth owing to the presence of the bacteria, and a marked retardation in the single infected culture which did grow. The experiment emphasises the need for prevention of contamination by bacteria, and for means for their detection.

Mortality of the cultures.

The only experiments that I have been able to find in which the growth of isolated yeast cells has been studied in small volumes of medium are those of R. J. Williams [1919]. In these the percentage of growing cells obtained seemed to be very low, especially in his control medium, which closely resembled the synthetic medium used in my experiments. I have therefore drawn up Table IV, in which are compared the percentages of growing cells obtained in Williams' experiments and my own.

Table IV.

	A. Single cells			B. Two cells			
Medium	Cultures considered	Number growing	% growing	Cultures considered	Number growing	% growing	Observer
Synthetic	95	25	27	85	32	38	Williams
Synthetic	21	16	76	43	30	70	Peskett
"Bios"-containing	210	113	54	164	105	64	Williams
	73	37	50	34	20	59	Peskett

It will be seen that the percentage of growing cells obtained in the synthetic media was considerably greater in my experiments than in those of Williams. The percentages for the "bios"-containing media in my experiments are unduly low as an earlier experiment has been included in which the mortality was high. If this experiment is omitted the percentages are, for the single cell cultures 66 %, and for the two-cell cultures 87 %, in the media containing "bios."

The technique employed in the later experiments, *i.e.* as described in this communication, is capable therefore of yielding a high percentage of growing cells.

Discussion.

The results obtained raise some points of interest.

In the first place the use of growth curves brings out clearly the variations, in some cases considerable, which may occur in the growth of individual cultures in spite of all precautions that may be taken to standardise the conditions of growth. Robertson [1923, p. 87] quotes a series of isolated observations of his cultures after a definite period of time as evidence that the growth of infusoria is approximately inversely proportional to the volume of culture medium employed. Such isolated observations appear to be of little value. For if we were to consider the growth at two definite times in the experiment shown in Fig. 1, say at 20 hours and at 70 hours, we should in the first case group together cultures 3, 1 and 4, and in the second case 5 and 6. In this way any significance of the relatively slow growth curve of culture 3 or of the relatively rapid growth of 5 would be lost. For this reason, observations of the growth have been taken at frequent intervals, and only complete growth curves have been considered in studying the effect of volume.

In the second place, it is interesting to compare the concentrations of yeast employed in these experiments with those used by Pasteur and Liebig, together with the growth obtained in each case. Pasteur inoculated "une tête d'épingle" of fresh yeast, containing 80 % water, into 100 cc. of medium and obtained a growth in one experiment amounting to 0·043 g. of yeast weighed dry. Assuming a pin's head to be approximately a sphere of 1 mm. diameter it would have a volume of about 0·5 mm.^{3}. Pasteur, then, inoculated about 0·1 mm.^{3} of actual yeast and this multiplied about 400 times.

Liebig using 100 cc. of medium inoculated with what he describes as "une trace de levûre." The only definition of this term that I can find occurs on p. 31 of his communication where he states "J'emploie de la levûre en pâte de la grandeur d'une tête d'épingle que je délaye dans 10 centimêtres cubes d'eau; je prends de celle-ci un centimêtre cube." Apart from any water that he may have used to render the yeast "en pâte" Liebig probably inoculated about one-tenth the amount of Pasteur, *i.e.* about 0·01 mm.^{3}. He failed to note any multiplication of the yeast.

If we can consider the yeast cell as a sphere of diameter about 10μ, and

therefore of about 5×10^{-7} mm.^{3} volume, then the range of volumes studied in my experiments was from 4×10^6 to 3×10^8 times the volume of a yeast cell in the synthetic media, and from 1×10^6 to 7×10^8 times in the media containing "bios" extracts. Single cells in these volumes would correspond to concentrations varying from 0·025 to 0·0003 mm.^{3} of yeast per 100 cc. of medium in the former and from 0·1 to 0·00014 mm.^{3} of yeast per 100 cc. in the latter experiments. On the synthetic media the growth obtained represented a multiplication of 5 to 28 times the amount inoculated, and on the "bios"-containing media from 120 to 18,000 cells were obtained from a single cell. On medium containing commercial cane sugar (Medium 1) multiplication of about 300 to 500 times was observed over a similar range of volumes.

It will be seen therefore that growth similar to that recorded by Pasteur was only obtained in the media containing "bios," although the range of volumes in both sets of experiments included values which, for the number of cells present, were comparable with those which Liebig and Pasteur employed. In none of my experiments was there any evidence that multiplication had been less in the larger volumes. On the contrary there was some evidence to show that the quickest growth occurred in the largest volumes, in the presence of "bios" extracts. The differences between the results obtained by Liebig and Pasteur cannot therefore be ascribed to differences in the volumes of medium used per yeast cell inoculated, unless the yeast employed by them was very different from that used in my experiments. They would appear to have been due to the presence of different amounts of "bios" in the cultures.

In this connection I have observed that the appearance of the cells has been similar to that described by Pasteur ("une belle levûre très ramifiée, extrêmement jeune d'aspect, c'est à dire que les globules sont gonflés, translucides, non-granuleux") only in the media containing "bios." In the synthetic media the growth was composed of very granular cells.

By further experiments in still larger volumes, I hope to elucidate the cause of the delay in multiplication observed in large volumes by Kuster [1907] and Wildiers [1901].

Thirdly, provided that the concentration of "bios" used has not been so great as to induce the "autostatic phase" of growth, the results obtained in the media containing "bios" are difficult to bring into line with the theory of an extracellular autocatalyst for growth suggested by Robertson [1923, pp. 102 *et seq.* and p. 147]. In all the experiments in Table II the growth was of a progressively increasing character, and therefore presumably in the "autokinetic phase."

In accord with Robertson's theory we should expect to find the reproductive rate enhanced in the smaller volumes and retarded in the larger, as the cells should at the time of division lose relatively less autocatalyst in the smaller volumes than in the larger. The results given above show that any enhancement of reproductive rate that may occur cannot definitely be attributed to a smaller or a larger volume, and that on the whole the evidence

points towards acceleration in the larger. In any case, Robertson's theory cannot be applied to the yeast used as no evidence of retardation of growth in larger volumes could be obtained.

Finally, the method employed includes a control of possible bacterial contamination, the importance of which has been demonstrated experimentally. Moreover, it yields a higher percentage of growing cells than that described by Williams. I hope that it may be usefully employed for the study of growth in other unicellular organisms.

SUMMARY.

1. A method is described for the study of the growth of single cells. Using this method, I have obtained a high percentage of living cells, and I have been able to detect readily those cases in which bacterial contamination has occurred.

2. The growth curves of one and two-cell cultures of yeast, grown in the absence of bacteria and in different volumes of medium, have been studied with the following results:

(*a*) In the absence of "bios." No constant effects on growth were observed as a result of differences in the volume of medium used.

(*b*) In the presence of "bios." The quickest growth occurred in larger volumes than the slowest, but there was not constant evidence to show that the growth was directly proportional to the volume. It was, however, definitely shown that the growth was not inversely proportional to the volume, as is the case in infusoria according to Robertson's observations.

3. The results summarised in 2 (*a*) and (*b*) are not in accord with Robertson's theory of an extracellular autocatalyst for growth.

4. The results obtained show that the differences between the observations of Pasteur and Liebig cannot be attributed to differences in the volumes of media employed by these workers per yeast cell inoculated, and that they were probably due to the presence of different amounts of "bios."

In conclusion I wish to express my indebtedness to Mr H. M. Carleton for his suggestion of the use of collodion in fixing the cultures and his advice throughout the elaboration of the bacteriological technique, and to Prof. Peters for his advice and interested criticism throughout the work. My thanks are also due to the Medical Research Council for a part time grant, and to the Government Grant Committee of the Royal Society for a grant to cover expenses.

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LXX. STUDIES ON THE GROWTH OF YEAST.

II. A FURTHER NOTE ON ALLELOCATALYSIS.

By GEOFFREY LEWIS PESKETT.

From the Biochemical Laboratory, Oxford.

Report to the Medical Research Council.

(*Received April 22nd, 1925.*)

In a recent communication Robertson [1924] has claimed that the failure of Cutler and Crump [1923] to observe allelocatalysis in cultures of *Colpidium colpoda* was due to the omission of the precaution of washing the organisms before inoculation. In my previous communication on the subject [1924] I failed to observe allelocatalysis in cultures of yeast, but I also had omitted to wash the yeast cells except in two of the experiments. In these two experiments, as stated at the time, some doubt existed as to whether the washing process had not actually added some organic matter to the culture owing to the technique employed. I have therefore repeated two typical experiments with the improved technique described in the preceding communication. Before suspending the cells in the required medium for the drop cultures, I washed them in the following manner.

Method.

The apparatus consisted of a large boiling tube which was plugged tightly with cotton wool at its open end. The plug was covered with fine-meshed muslin to prevent pieces of it from becoming detached and drawn down into the medium during the centrifugalisation. The edges of the muslin were firmly attached to the rim of the tube to keep the plug in position. Through the plug and muslin passed a Dreyer's pipette also plugged at its wider end. In the boiling tube were placed five small test tubes of narrow bore, separated from the bottom of the tube by a small pad of cotton wool. These tubes bore permanent identification marks so that they could be readily distinguished from each other. Before filling them with medium the whole apparatus was thoroughly cleansed and sterilised. From 2·0 to 2·5 cc. of the sterile synthetic medium (Medium 2) were then placed in each of the tubes under aseptic conditions, and the whole was again sterilised by steam heat in the Koch steriliser for 45 minutes on three successive days.

About 0·25 cc. of the culture containing the cells to be washed was admitted to the apparatus by a capillary pipette inserted into the Dreyer

pipette. The plug was replaced with the usual precautions and a rubber teat was used to force the fluid containing the cells down into the first tube of synthetic medium, which was then gently agitated by passing a few bubbles of air through it. After standing a few minutes the whole apparatus was placed in a large centrifuge. The cells, which in the case of the yeast used sink very readily, were brought to the bottom by 30 to 45 seconds' centrifugalisation at about 600 r.p.m. The supernatant fluid was now pipetted off and a fresh amount of medium introduced from the next tube. The washing and centrifuging were then repeated.

It was found that the cells could be very thoroughly washed by this method with only the slightest risk of infection, provided that the apparatus was so arranged and care was taken to see that the Dreyer pipette was only moved in the wool plug as little as possible, in both horizontal and vertical directions. Moreover, the mortality among the washed cells was 50 % in Medium 1 and 28 % in Medium 2—figures which compare favourably with those for the previous experiments in which the cells were not washed.

Results.

By the technique described above, the cells used in these experiments were washed five times with from 2·0–2·5 cc. of synthetic medium. Assuming that one-tenth of a cc. of medium was left after each washing, then the concentration of any extraneous matter introduced with the cells in the first instance was reduced after washing five times to less than 1/3,000,000. At the end of the washing process we can assume, therefore, that we are dealing with yeast cells in synthetic medium free from any impurities other than those which may be present in the medium itself owing to the difficulty of preparing absolutely pure chemicals.

After washing, some of the cells were suspended in synthetic medium (Medium 2), others in the medium containing commercial cane sugar (Medium 1). The results are given in the following table in which is recorded the number of cells present after a given time in cultures which contained originally one, two or three cells. The cultures were grown at 25° and observations taken as described in Part I.

Exp. 10, Medium 1. Growth after 34 hours at 25°.

1 cell	2 cells
10, 8	9, 12, 9, 19

The corresponding drop volumes were, in mm.^{3},

103, 23	23, 16, 16, 16

Exp. 11. Medium 2. Growth after 45 hours at 25°.

1 cell	2 cells	3 cells
3, 5, 4, 2	10, 7, 8, 3, 5, 4, 5	9, 6

The corresponding drop volumes were, in mm.^{3},

106, 23, 106, 19	10, 17, 23, 19, 17, 17, 51	31, 12

No cultures in these experiments have been excluded from the table on account of exceptionally high growth, and all were examined after fixing and staining and found to be free from bacteria. The absence of any effect which could be termed allelocatalytic was confirmed at all the other periods at which the cultures were examined, as well as at the times recorded in the table. The results show that allelocatalysis does not occur in the case of the yeast studied even after washing the cells before inoculation.

Conclusion.

The failure to observe allelocatalysis in the experiments recorded previously was not due to the omission of the precaution of washing the cells before inoculation. My previous conclusion was therefore justified, namely, that allelocatalysis does not occur in the case of the yeast studied.

My thanks are due to the Medical Research Council for a part time grant, and to the Government Grant Committee of the Royal Society for a grant to cover expenses.

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LXXI. FERMENTATION BY DRIED YEAST PREPARATIONS.

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Some years ago the behaviour of zymin (yeast dehydrated by acetone) with different volumes of sugar solution was described by Harden and Young [Harden, 1911, p. 37].

When zymin is incubated with 10 cc. of approximately 10 % glucose solution at 25°, fermentation commences almost immediately, increases to a maximum rate in about 40 minutes and then very slowly decreases in rate. If, however, 25 cc. of the glucose solution of the same concentration be employed with the same weight of zymin, only a very slow fermentation occurs for the first hour and a half, after which the rate increases and finally becomes only slightly less than that in the presence of 10 cc. of the glucose solution. The curves *A* and *B* in Fig. 1 show this behaviour in typical manner, but the actual volume of gas evolved varies with different samples of zymin. A similar phenomenon is shown by dried yeast (Fig. 2) but the difference in behaviour is often still more marked. When larger volumes of glucose solution are taken the onset of fermentation is still further delayed or may even be entirely prevented. This is shown by the following results:

A. 1 g. zymin + 5 cc. of 10 % glucose solution + 0·2 cc. toluene.

B. 1 g. zymin + 20 cc. of 10 % glucose solution + 0·2 cc. toluene.

Fermentation commenced at once in A, at the rate of about 0·6 cc. per 5 minutes, and at the end of 7 hours 66 cc. of CO_2 had been evolved, whereas no fermentation was observed in B, even after 23 hours.

The autofermentation of zymin is affected in a similar manner. Thus 2 g. zymin (1) + 10 cc. H_2O, and (2) + 25 cc. H_2O at 25° gave the results shown in curves *A* and *B*, Fig. 3.

These observations have evidently an important bearing on those discussed by Abderhalden and Fodor [1921] and subsequently studied by Sobotka [1924].

The thesis maintained by Abderhalden and Fodor is that the complex chemical action of yeast on sugar is only reproduced by preparations such as acetone-yeast (zymin), yeast-juice, maceration extract or dried yeast, in so far as the original colloidal condition of the protoplasm of the yeast cell is retained. Without at the moment discussing this interesting and important conclusion, for and against which much can be said, attention may be directed to one of the arguments adduced which appears to be founded on a misunder-

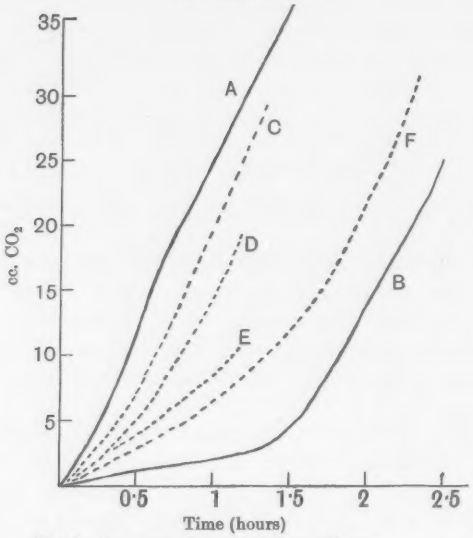

Fig. 1. 2 g. zymin in each + 0·2 cc. toluene.

A. 10 cc. of 10 % glucose } Solutions added direct.
B. 25 cc. „ }
C. 25 cc. „ ; 0·2 *M* $NaC_2H_3O_2$ } Solutions added after preliminary moistening for 1 minute.
D. 25 cc. „ ; 0·1 *M* „ }
E. 25 cc. „ ; 0·05 *M* „ }
F. 25 cc. „

(The curve for 0·025 *M* acetate is identical with *E*.)

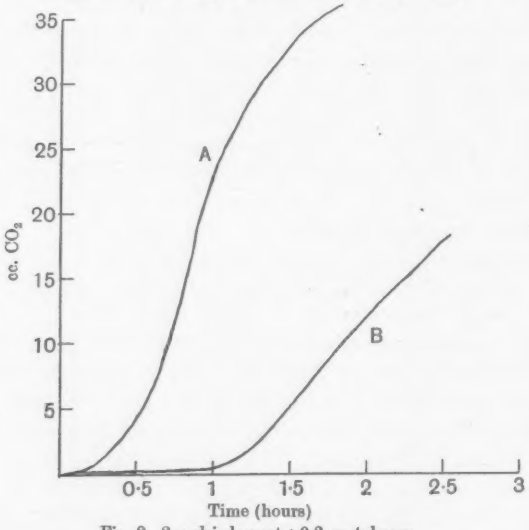

Fig. 2. 2 g. dried yeast + 0·2 cc. toluene.

A. + 10 cc. of 10 % glucose } added direct.
B. + 25 cc. „ }

standing. One of the marked differences between living yeast and yeast preparations is alleged to be that these preparations are incapable of fermenting dilute sugar solutions (p. 143). "Ebensowenig wie Hefeauszüge vermag sterile Trockenhefe (Acetondauerpräparat oder durch Alter sterilisierte Hefe) verdünnte Zuckerlösungen zu vergären, weil allen diesen Stoffen die Fähigkeit der Konzentration, die nur lebenden Zellen eigen ist, fehlt." The statement appears to be founded on the fact that Buchner [1903, p. 260] used 40 % cane sugar in his fermentation experiments with zymin, and only in one instance published results obtained with another concentration. Buchner, moreover, used 4 g. cane sugar and 10 cc. of water to 2 g. zymin (Dauerhefe), a proportion which gives almost immediate fermentation.

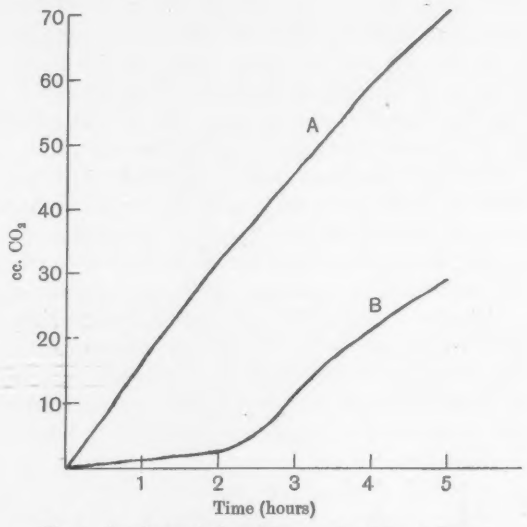

Fig. 3. Autofermentation of 2 g. zymin + 0·2 cc. toluene.
A. +10 cc. H_2O } added direct.
B. +25 cc. H_2O }

Actually, more dilute solutions of glucose are fermented by zymin more rapidly than 40 % solutions, provided that only a small volume of the solution is used.

In Table I are given figures for 10 cc. and 25 cc. respectively of each of the concentrations 2·5, 5, 10, 20 and 40 g. of glucose per 100 cc. with 2 g. zymin.

Abderhalden and Fodor made experiments using 10 g. dried yeast, 10 g. sugar and 250 cc. of H_2O and it is therefore not a matter for surprise that they obtained long induction periods, followed by fermentation due to the growth of yeast cells.

Table I.

2 g. zymin with (*a*) 10 cc. glucose solution + 0·2 cc. toluene } at 25°.
(*b*) 25 cc. glucose solution + 0·2 cc. toluene }

cc. of CO_2 evolved.

Time of incubation (mins.)	(*a*) 10 cc. Concentration, g. per 100 cc.					Time of incubation (hrs.)	(*b*) 25 cc. Concentration, g. per 100 cc.				
	2·5	5	10	20	40		2·5	5	10	20	40
10	1·8	3·3	2·1	2·5	2·5	1	2·7	2·2	3·6	3·6	4·5
20	4·4	8·1	5·1	5·2	4·5	2	3·0	2·7	7·8	7·7	6·4
30	8·4	12·5	8·7	7·9	5·9	3	15·0	11·1	17·8	16·2	8·7
40	12·8	16·0	11·6	10·6	7·2	8	71·4	58·1	64·5	47·8	30·0
50	16·5	20·0	14·0	13·7	8·4						
60	19·9	24·6	16·5	15·9	9·9						
130	43·8	50·1	38·1	32·8	19·8						

Sobotka, who has more recently taken up the study of fermentation by dried yeast [1924] has carried out further work on the same lines as Abderhalden and Fodor and has come to the conclusion (which he also ascribes to them, erroneously as it seems to me) that the effect produced by dried yeast is to be attributed exclusively to living cells; "die Leistung der Trockenhefe ausschliesslich auf lebende Zellen zurückzuführen ist" (p. 9).

Sobotka used 1 g. of dried yeast with 1 g. glucose and 20 cc. of water and observed periods of induction of 80–260 minutes with one group of yeasts and 4–19 hours with another group. Only one experiment on the effect of yeast concentration is recorded. In this the quantity of yeast per 20 cc. of 5 % glucose was varied and the resulting very marked decrease in the incubation period explained as being due to the shorter time required for the production of the necessary number of cells capable of fermenting.

There seems to be little doubt that Sobotka's results were actually due to the growth of living yeast cells contained in his preparations but his general conclusion is too sweeping. The "Dauerhefe" prepared by Buchner and Albert was carefully tested by them and found to be free from yeast cells capable of growth and yet they attained good and immediate fermentation by its use. Three successive samples of zymin prepared in this laboratory were also found to be free from yeast cells capable of growth, so that the undoubted fermentative powers of these samples must be attributed to the enzyme contained in the dead cells. Now ordinary dried yeast, which admittedly contains living yeast cells, behaves in an analogous manner to zymin when brought into different volumes of sugar solution and it is therefore only reasonable to attribute part at least of the fermentation produced by dried yeast to the enzyme in the dead cells.

No entirely satisfactory explanation of the phenomenon has as yet been found. The facts suggest that the problem, as suggested by Abderhalden and Fodor [1921], is concerned with the dispersion of the enzyme. If we suppose that this occurs rapidly in a more concentrated solution of the extractives of zymin, less rapidly in a more dilute solution, the long induction period in a large volume of sugar solution would be explained. In favour of

this view is the fact that the onset of fermentation can be greatly hastened in two different ways, each of which is consistent with the foregoing suggestion, viz. by

(1) The addition of a boiled extract of the dried yeast.

(2) The addition of certain salts, *e.g.* sodium acetate or sodium bicarbonate.

On the other hand the action of these salts is to some extent specific and experiments still in progress indicate that washing zymin with sodium acetate solution does not remove the enzyme any more rapidly than does washing with distilled water, as would be expected if it caused rapid dispersion of the enzyme. Various other possible explanations suggest themselves and these are now being investigated.

The effect of an extract of the dried yeast and of sodium acetate or bicarbonate solution is exhibited in the following experiments.

A. *Extract of zymin or of dried yeast.*

(1) *Zymin.*

2 g. of zymin + 0·2 cc. toluene were treated at 25° in each case with (1) 1 g. glucose + 10 cc. H_2O; (2) 1 g. glucose + 10 cc. of an extract of 4 g. zymin in 20 cc. of boiling water; (3) 2·5 g. glucose + 25 cc. H_2O; (4) 2·5 g. glucose + 25 cc. of an extract of 4 g. zymin in 50 cc. boiling water. The resulting solutions of yeast extractives in (2) and (4) were therefore approximately twice the concentrations of those in (1) and (3). As seen from the figures showing the course of fermentation, the addition of extract hastened the onset of fermentation slightly in the case of 10 cc., very considerably in that of 25 cc. and further, as would be expected, increased the rate of fermentation owing to the additional phosphate and co-enzyme added.

	cc. CO_2 evolved.			
	2 g. zymin + 1 g. glucose		2 g. zymin + 2·5 g. glucose	
Time (hours)	10 cc. H_2O	10 cc. extract	25 cc. H_2O	25 cc. extract
0·25	1·4	7·7	0	2·8
0·5	5·6	17·6	0	5·2
1·0	16·5	31·8	1·5	11·2
2·0	36·4	52·5	10·5	23·9
3·0	—	—	28·8	46·8

(2) *Dried yeast.*

(*a*) 2 g. dried yeast + 2·5 g. glucose + 0·2 cc. toluene in each case: (1) + 25 cc. H_2O; (2) + 25 cc. extract made by heating 4 g. dried yeast with 50 cc. H_2O in a boiling water-bath for 17 minutes. No fermentation was produced in 20 hours in the absence of extract; whereas in its presence fermentation commenced after an hour and a half.

(*b*) 2 g. dried yeast + 1 g. glucose + 0·2 cc. toluene in each case: (1) + 10 cc. H_2O; (2) + 10 cc. extract made by heating 4 g. of dried yeast with 20 cc. H_2O in a boiling water-bath.

Time (hours)	cc. CO_2 evolved. 2 g. dried yeast + 2·5 g. glucose: 25 cc. H_2O	25 cc. extract	2 g. dried yeast + 1 g. glucose: 10 cc. H_2O	10 cc. extract
0·25	0	0·2	0	1·3
0·5	0	0·7	0·2	3·2
1·0	0	1·5	0·7	8·6
1·5	0	3·6	3·8	16·2
2·0	0	6·9	10·2	23·2
3·0	0	17·0		

B. *Sodium acetate.*

Preliminary experiments showed that the substitution of 0·2 *M* $NaC_2H_3O_2$ for water caused a great diminution of the "period of induction" without serious change of p_H and further investigation showed that the effect was a maximum at about 0·2–0·3 *M* concentration of the acetate. No analogous effect could be produced with sodium chloride. Some irregularity was at first obtained in the results with different concentrations, but it was found that this could be obviated by adding to the 2 g. of zymin 5 cc. of water, shaking for 1 minute and then adding 15 cc. of a solution containing 2·5 g. of glucose and 5 cc. of acetate of the requisite concentration.

Typical fermentation curves for zymin with a series of concentrations of $NaC_2H_3O_2$ are given in Fig. 1, *C*, *D*, *E*, *F*. Similar results were obtained with dried yeast.

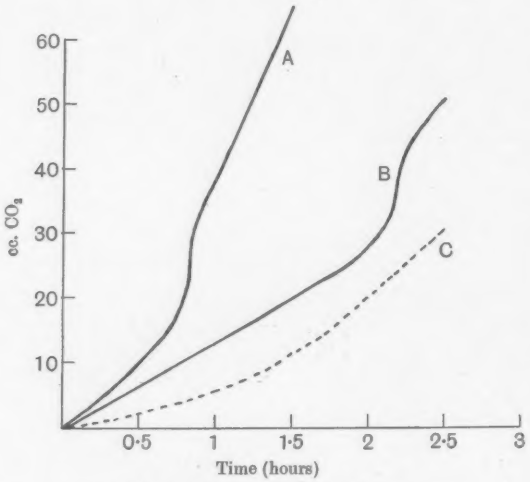

Fig. 4. 2 g. zymin + 0·2 cc. toluene.

A. +25 cc. of 10 % glucose; 0·05 *M* $NaHCO_3$
B. " " ; 0·1 *M* $NaHCO_3$
C. " "
} Solutions added after preliminary moistening for 1 minute.

C. *Sodium bicarbonate.*

Sodium bicarbonate produces a similar effect, the most favourable concentration, under the conditions employed above, being 0·1 *M*. Regular results were only obtained by adopting the same method of adding the solutions as was used for the acetate (Curves *A* and *B*, Fig. 4). An interesting point of difference between the curves obtained with acetate and bicarbonate is that the latter show a pronounced "phosphate effect" whereas the former do not.

Summary.

1. Air-dried yeast and zymin (yeast dehydrated by acetone) produce fermentation rapidly in a small volume of sugar solution, only after considerable delay in a large volume.

2. The addition of yeast extract or solutions of certain salts such as acetates or bicarbonates greatly accelerates the onset of fermentation in presence of a large volume of sugar solution.

3. Air-dried yeast possesses a definite fermenting power which is not dependent on the presence of living cells.

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LXXII. THE ESTIMATION OF PHOSPHORUS AND MAGNESIUM.

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(*Received April 29th, 1925.*)

I. Phosphorus.

In the estimation of phosphorus, the highest accuracy, undoubtedly, is to be attained by the use, in conjunction with a delicate micro-balance, of some gravimetric method in which the phosphorus is precipitated as a compound of which it forms only a small percentage. Gravimetric methods, however, are apt to be long and troublesome. Particularly is this the case when the amount of substance to be estimated is very small, so that slightly incomplete drying of the precipitate, for example, may introduce a very considerable percentage error into the final result. Although the colorimetric method described by Bell and Doisy [1920]—especially in the modified form introduced by Briggs [1922]—is rapid and convenient, we have had difficulty in obtaining uniformly accurate results by its use, except in the case of inorganic phosphorus. The main sources of error in the estimation of acid-soluble and total phosphorus have been pointed out by Martland and Robison [1924]. They reduce the amount of sulphuric acid used in the ignition since they find that the amount used by Briggs, with that added in the molybdate solution, may bring the total acidity near, or even above, the limit of safety. Further, they allow the ignition to continue for as short a time as possible, to prevent loss of phosphorus by absorption by the glass.

Another source of error, which applies to estimations of inorganic phosphorus, is introduced by the trichloroacetic acid, which these authors find to contain an impurity, which itself gives a blue colour with the Briggs reagent. This error is serious only when the colorimetric readings are made after long standing, and the interfering substance may be removed by distilling the trichloroacetic acid. Although, by incorporating these modifications in the method, accuracy may be attained, we desired to avoid, if possible, the use of colorimetry, and therefore turned our attention to volumetric methods.

It has long been customary, in determining the phosphorus content of steel, etc., to precipitate the phosphorus as ammonium phosphomolybdate, to dissolve the precipitate in standard sodium hydroxide solution, and to titrate the excess alkali with standard acid. This method, which was originated

by Pemberton [1893], is very well known. The amounts of phosphorus dealt with, however, have been much greater than those present in the small volume of blood available for analysis, and it was therefore our first task to see whether the method was sufficiently delicate to estimate with reasonable accuracy the 0·05 mg. of inorganic phosphorus present in 2 cc. of normal blood. This, once the appropriate conditions of precipitation and titration had been determined, was found to be the case; it was possible to estimate, within 3 %, 0·05 mg. of phosphorus contained in 20 cc. of fluid. A second series of experiments showed that exactly the same conditions with respect to quantity of precipitant, etc., sufficed for the estimation of six times that concentration of phosphorus with, indeed, somewhat greater accuracy. These results (Table I) showed that the method was capable of estimating the inorganic phosphorus in 2 cc. of blood through a range extending from below to far above the normal value. For total, or acid-soluble phosphorus, even less blood would be required.

Table I. *Estimation of phosphorus in a solution of pure potassium dihydrogen phosphate.*

Weight of phosphorus in mg.		
Present	Found	Error %
0·05	0·0514	3
	0·0485	3
	0·0505	1
	0·0497	0·6
0·10	0·0999	—
	0·101	1
	0·101	1
	0·0995	0·5
0·15	0·150	—
	0·152	1
0·20	0·198	1
	0·199	0·5
	0·199	0·5
	0·201	0·5
0·30	0·298	0·6
	0·305	1·6
	0·302	0·6
	0·297	1

There remained a further important point to be determined. Bell and Doisy [1920] have pointed out that in their blood filtrate the inorganic phosphate increases on standing, becoming, after the lapse of a few hours, equal to the acid-soluble phosphorus, owing to the gradual hydrolysis in acid solution of the organic phosphorus-containing substances present. Martland and Robison [1924], on the other hand, have carried out experiments which appear to show that the hydrolysis is enzymic and practically ceases after the addition of trichloroacetic acid. Hence they recommend that the laking of the blood and the precipitation of proteins by trichloroacetic acid (1 %) be carried out as quickly as possible, whilst Bell and Doisy consider it essential that the whole estimation be carried out at once. In face of these contradictory results, we considered it necessary to determine with what rapidity

the estimation must be carried out. For our experiments on the point to have any force, however, the precipitation of ammonium phosphomolybdate must be complete in a few minutes so that the estimation may be carried out rapidly; and equally, on longer standing, there must be no further precipitation—*e.g.* of ammonium tetramolybdate or of molybdic acid. That this condition is amply satisfied is shown by the results recorded in Table II, from which it appears that the precipitation of phosphorus from a solution of potassium phosphate is complete in 10 minutes, and is not increased when the reaction mixture is allowed to stand for as long as 14 hours. The second part of Table II gives the results of our experiments on the time factor in the case of blood. It will be seen that they confirm the results of Martland and Robison [1924], showing that, whilst it is necessary to precipitate the blood proteins as rapidly as possible after laking, the amount of inorganic phosphate in the protein-free acid filtrate undergoes little or no change on standing.

Table II.

(*a*) *Estimation of phosphorus in a solution of pure potassium dihydrogen phosphate.*

Time allowed for precipitation	Weight of phosphorus in mg.	
	Present	Found
5 mins.	0·20	0·12
10 „	0·20	0·199
15 „	0·20	0·198
	0·20	0·201
20 „	0·20	0·201
	0·20	0·201
30 „	0·20	0·199
60 „	0·20	0·201
14 hours	0·20	0·201
	0·20	0·199

(*b*) *Estimation of inorganic phosphorus in blood.*

	mg. P/100 cc.
I. Blood laked and proteins precipitated at once. Inorganic phosphorus estimated in filtrate at once. 15 mins. allowed for precipitation of ammonium phosphomolybdate	3·86
Inorganic phosphorus estimated at once. 2 hours allowed for precipitation of ammonium phosphomolybdate	3·86
Inorganic phosphorus estimated after allowing protein-free filtrate to stand 2 hours at room temperature. 15 mins. for precipitation of ammonium phosphomolybdate	3·90
II. Blood laked and allowed to stand 1 hour at room temperature. Proteins then precipitated and inorganic phosphorus estimated in filtrate at once. 15 mins. for precipitation of ammonium phosphomolybdate	4·60

Still further preliminary experiments with known amounts of phosphorus showed that the accuracy of the method was not affected by the presence of such substances as are either found normally in blood and urine or are added in preparing them for analysis. There was no loss of phosphorus during ignition with sulphuric and nitric acids for the removal of organic matter (Table III).

Table III.

(*a*) *Potassium dihydrogen phosphate added to urine. Inorganic phosphorus estimated.*

Weight of phosphorus added in mg./100 cc.	Weight of phosphorus found in mg./100 cc.	% of added phosphorus recovered
—	63·0	—
32·2	95·8	101·8
61·7	124·2	99·2
105·5	168·0	99·5

(*b*) *Salts etc. added to a solution of pure potassium dihydrogen phosphate.*

	Weight of phosphorus in mg.	
Substance added	Present	Found
Sodium sulphate (2·5 g.)	0·10	0·099
Trichloroacetic acid (0·4 g.)	0·20	0·199

(*c*) *Solution of pure potassium dihydrogen phosphate, with added cane sugar ignited with the sulphuric-nitric acid mixture.*

Weight of phosphorus in mg.	
Present	Found
0·10	0·101
0·20	0·199

Method.

20 cc. of the liquid to be examined were measured into a 100 cc. beaker, and 1 cc. concentrated nitric acid and 3 cc. 32 % ammonium nitrate were added. The beaker was heated t 70–75° on the water-bath, and, after the addition of 5 cc. 3·5 % ammonium molybdate, was re-heated to that temperature. It was then allowed to stand and cool. After not less than 10 minutes —we adopted 15 as the standard time—the liquid was filtered through paper pulp which should be free from scraps of unpulped paper. We have found that the presence of such scraps hinders very considerably the complete solution of the precipitate in the standard alkali. In the filtration, only a moderate suction should be used. The precipitate was then washed, first with a 2 % solution of nitric acid, and finally with a 2 % solution of potassium nitrate until the washings were free from acid. Usually, two washings with acid and four with potassium nitrate were sufficient, using 20 cc. for each washing. The paper pulp and precipitate were then returned to the beaker, the funnel and glass rod being washed with the potassium nitrate solution, and not with distilled water, which decomposed the precipitate giving high values. The precipitate was then dissolved in a known volume of standard sodium hydroxide, and the excess alkali determined by titration with standard acid.

The indicator used in the titration was phenolphthalein, and it was therefore necessary to use CO_2-free alkali. Moreover, since shaking to dissolve the precipitate in the open air inevitably caused the absorption of an appreciable —and varying—amount of CO_2, it was necessary to carry out the titration in

a CO_2-free atmosphere. The acid and alkali (CO_2-free) were kept in paraffined bottles from which they were siphoned into burettes by means of the arrangement described by Cole [1920]. The delivery tubes of the burettes passed through holes in a rubber stopper which fitted the mouth of the beaker used in the precipitation. Through this stopper passed also a third, small, burette, from which the indicator was added, and two glass tubes by means of which a stream of air was drawn through the beaker during the titration. Carbon dioxide was removed from the air by means of soda-lime. The air inlet tube reached almost to the bottom of the beaker so that the air current not only removed CO_2 from the potassium nitrate solution used to wash in the precipitate, but kept the liquid well stirred. The beaker containing the precipitate was placed in position and the air current was started. Two minutes sufficed to remove CO_2 from the potassium nitrate solution and from the air in the beaker. 10 cc. $N/70$ sodium hydroxide were then added from the (20 cc. capacity) burette. The precipitate dissolved readily unless the quantity of ammonium phosphomolybdate was too great, in which case a second 10 cc. were added. As soon as the precipitate was completely dissolved, a drop of phenolphthalein was added, and the excess alkali was titrated with $N/70$ sulphuric acid.

1 cc. $N/70$ sodium hydroxide used in dissolving the ammonium phosphomolybdate was equivalent to 0·0198 mg. phosphorus. The factors given in the literature vary enormously, for though the precipitate is here referred to as ammonium phosphomolybdate its composition is not by any means constant but appears to depend on the conditions under which it is formed. Whatever its actual composition may be, however, a large number of determinations has shown that it does not vary when the reasonably elastic conditions we prescribe are adhered to. The factor given above is based on our own work.

By the use of still more dilute standard solutions it would, no doubt, be possible to achieve greater accuracy in dealing with the very small amounts of inorganic phosphorus met with in normal blood, but we chose $N/70$ solutions for two reasons. Firstly, they cover adequately the required range, since with 0·05 mg. phosphorus the maximum error is only 3 % and the accuracy increases with increasing amounts of phosphorus up to 0·30 mg. Secondly, we employ solutions of this strength for other routine purposes such as the estimation of non-protein nitrogen and urea in blood.

Since the work described in this paper was completed, our attention has been called to a somewhat similar method for the estimation of phosphorus in urine and faeces [Richards and Godden, 1924]. These authors, however, deal with amounts of phosphorus vastly greater than those available in blood analysis. To overcome the difficulty of absorption of CO_2 by the alkali, they dissolve the precipitated ammonium phosphomolybdate in standard alkali (0·5 N), boil, add a known volume of standard acid in excess of that required for neutralisation and boil to remove CO_2. After cooling, the solution is titrated with standard alkali. Not only is this procedure longer than our

method of titration in a CO_2-free atmosphere, but it does not appear to us to be equally satisfactory for work on the micro scale, since it does not give so rigorous an exclusion of carbon dioxide.

Preliminary treatment of blood and urine.

Urine. 5 cc. urine were diluted to 250 cc. with distilled water. For *inorganic phosphorus*, 20 cc. of this solution were used and precipitated direct. For *total phosphorus*, 20 cc. were evaporated to small bulk in a wide boiling tube. 2 cc. of a mixture of three parts of concentrated nitric acid and one part of concentrated sulphuric acid were then added, and the mixture heated until nitrous fumes ceased to be evolved and white fumes of sulphur trioxide were just beginning to appear. When necessary, more nitric acid was added and heating continued in the same way until, after removal of the nitric acid, the residual solution was colourless. This was diluted with about 5 cc. distilled water and neutralised (to methyl orange) with concentrated sodium hydroxide solution. The neutral solution was then transferred quantitatively to a 100 cc. beaker for precipitation. The total volume with the washings was about 25 cc. *Organic phosphorus* was estimated by difference, but where the increased accuracy of a direct determination is required, the procedure of Taylor and Miller [1914] may be carried out. To 20 cc. urine in a 25 cc. flask they add powdered barium hydroxide until the liquid is just alkaline, make up the volume to 25 cc. with water and filter. To 20 cc. of the filtrate in another 25 cc. flask, they add dilute sulphuric acid until the liquid is slightly acid, fill up to the mark with water, and filter from the precipitated barium sulphate. 1 cc. of this second filtrate is equivalent to 0·64 cc. of the original urine. For the volumetric determination of organic phosphorus it should be diluted and treated as described above for total phosphorus. The determination of inorganic or organic phosphorus should be carried out on fresh urine.

Blood. For the determination of *total phosphorus* in blood, 0·5 cc. was treated with 2 cc. of the sulphuric-nitric acid mixture as described for urine. For *inorganic* and *acid-soluble phosphorus* 5 cc. blood were laked as quickly as possible by adding to 30 cc. distilled water in a 50 cc. flask. 5 cc. of a 25 % solution of trichloroacetic acid were added at once, and the liquid was diluted to 50 cc. After standing for 5 minutes it was filtered. 20 cc. of the filtrate (equivalent to 2 cc. blood) were used for the direct precipitation of the inorganic phosphorus; 10 cc. were treated as described for total phosphorus in urine for the determination of the total acid-soluble phosphorus.

A few typical results with blood and urine are given in Table IV. The colorimetric method used for comparative purposes was that of Briggs [1922] as modified by Martland and Robison [1924].

Table IV.

(1) *Blood.*

Phosphorus; mg./100 cc.					
Inorganic		Acid soluble		Total	
Colorimetric	Titrometric	Colorimetric	Titrometric	Colorimetric	Titrometric
3·75	3·76	26·60	26·40	34·4	34·8
3·40	3·33	23·64	23·59	—	—
4·40	4·43	22·20	22·10	—	—
3·28	3·23	25·20	25·00	—	—
2·96	2·99	22·20	22·08	—	—
3·35	3·34	23·40	22·97	—	—
3·28	3·31	26·67	26·83	35·8	35·5
(2) *Urine.*					
69·65	69·53	—	—	72·36	72·20
41·66	41·85	—	—	43·55	43·68

II. Magnesium.

Many of the methods at present in use for the estimation of magnesium in blood and serum depend on the estimation—usually colorimetric—of the phosphorus content of ammonium magnesium phosphate. It is therefore obvious that our method for the determination of phosphorus affords a means of estimating magnesium titrometrically. The average normal magnesium content of blood serum is 2·5 mg. per 100 cc.; 2 cc. serum thus contain 0·05 mg. magnesium, which corresponds to 0·129 mg. phosphorus, and is therefore well within the range of our method.

In dealing with serum, calcium is first precipitated as described by Kramer and Tisdall [1921]. 2 cc. serum are added to 2 cc. water in a 15 cc. centrifuge tube; 1 cc. of a saturated solution of ammonium oxalate is added, and the volume made up to 6 cc. with distilled water. After standing for half an hour, the precipitated calcium oxalate is removed by centrifuging. From 5 cc. of the supernatant liquor, magnesium is precipitated by the method of these authors. 1 cc. of a solution of ammonium phosphate and 2 cc. concentrated ammonia (0·880) are added and the mixture is allowed to stand overnight. (The solution of ammonium phosphate is prepared by dissolving 25 g. di-ammonium hydrogen phosphate in 250 cc. water, adding 25 cc. concentrated ammonia, allowing to stand overnight, filtering, boiling off excess ammonia, and diluting to 250 cc. For use, 20 cc. of this solution are diluted to 100 cc.) Next morning, the liquid is centrifuged and the precipitate washed by centrifuging three times with 5 cc. ammonia solution (10 cc. concentrated ammonia in 100 cc.). The precipitate is then dissolved in 5 cc. 2 % nitric acid; the solution is transferred quantitatively to a 100 cc. beaker, and the phosphorus estimated as usual. 1 cc. $N/70$ sodium hydroxide is equivalent to 0·0151 mg. magnesium. Table V gives a few results from standard solutions of magnesium sulphate and from blood serum.

Table V. *Estimation of magnesium.*

(1) In a solution of pure magnesium sulphate mg. magnesium per 100 cc.		(2) Found in serum mg. magnesium per 100 cc.
Present	Found	
1·25	1·22	I. 2·41
	1·23	II. 2·38
2·50	2·46	III. 2·49
	2·47	IV. 2·40
5·00	4·95	V. 2·52
	4·93	

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LXXIII. CONTRIBUTIONS TO THE STUDY OF BRAIN METABOLISM.

I. CARBOHYDRATE METABOLISM. PRELIMINARY PAPER.

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(*Received April 28th, 1925.*)

The work about to be described constitutes the preliminary portion of an investigation upon the metabolism of brain cells under conditions both normal and abnormal. The work of Warburg, Posener and Negelein [1924], who showed that brain tissue is capable of converting large amounts of glucose into lactic acid, suggested that a study of the sugar metabolism of the brain might be of considerable interest. It was therefore decided to attempt a comparison of sugar metabolism of the brain in a normal animal, and in an animal suffering from the effects of a convulsant dose of insulin. Rabbits were used for all the experiments.

When this work was already in progress, the papers of Takahashi and Asher [1924] and of Cori [1925] appeared. Takahashi dealt particularly with the total carbohydrate content of the brain, including glycogen, and Cori with the free sugar content. Both were agreed that the lowering of the blood sugar by insulin had very little effect upon the content of reducing substance in the brain.

Takahashi estimated glycogen and "other carbohydrate" (the latter being extracted by a modification of Meyerhof's method) in rabbit brains. The values for reducing substance were obtained by the Folin-Wu technique. His average value for normal animals was: glycogen 38·6, "other carbohydrate" 39·6 mg. %. For insulin-treated animals, prior to convulsions, the average values were: glycogen 46·5, "other carbohydrate" 42·5 mg. %; for "convulsed" animals, he gives three sets of values: glycogen 27, "other carbohydrate" 8; glycogen 6, "other carbohydrate" 41; glycogen 6, "other carbohydrate" 38 (all mg. %).

Cori treated his extracts of rabbit brain with Lloyd's alkaloidal reagent, with the object of removing reducing substances other than glucose, and then estimated the remaining reducing substance by the method of Hagedorn and Jensen. His figures for normal animals (used as controls) range from 43–25 mg. %. He gives only a few values for rabbits, and the variations

between them are large, yet length of time of starvation seems to be the only factor determining the selection of "control" animals, since these do not correspond with the "experimental" in age or weight.

We were able to confirm the fact that no alteration in the amount of reducing substance in the brain need necessarily have taken place at the onset of insulin convulsions. The actual figures we obtained were, however, higher than those given by the two authors mentioned. It is therefore thought advisable to give an account of the methods employed in this work.

The rabbit is killed by a blow on the neck, its brain removed as quickly as possible, and, where an immediate determination is to be carried out, the brain is then placed in a beaker surrounded by freezing mixture, chopped whilst very cold, and weighed out when frozen hard. Whilst still frozen it is placed in 20 % trichloroacetic acid (or alternatively in absolute alcohol). The removal of the brain can usually be accomplished in four or five minutes.

Two methods of extraction were employed, and where possible were both carried out on the same brain.

I. Extraction with trichloroacetic acid (20 or 10 %), a method suggested to us by Mr H. D. Kay. The tissue is ground with sand and ice-cold trichloroacetic acid, allowed to stand for some minutes at 0°, and filtered through muslin. This process is carried out three times. The cloudy solution thus obtained is centrifuged (filtration being very slow or practically impossible), the supernatant fluid poured off, and the precipitate washed three times before being discarded. The beakers, muslin, etc., are also well washed. The solution is next neutralised. It is usually still faintly cloudy, but a final centrifuging after neutralisation leaves it absolutely clear. The reducing substance present in this clear solution is estimated by the method of Hagedorn and Jensen [1923].

II. Extraction with alcohol. The tissue is allowed to soak in 90 % alcohol for some minutes to stop all enzyme action. It is then filtered off through muslin, ground up, placed in 60 % alcohol and left overnight. Next day the tissue is filtered off, ground, soaked for an hour in 60 % alcohol and finally filtered. The process is repeated once more. The cloudy filtrate is centrifuged till it is as clear as possible, the supernatant fluid poured off, and the precipitate washed three times before it is discarded. The solution is next heated gently on a water-bath until all the alcohol has disappeared; it is then full of a flocculent precipitate. Colloidal "iron" is added until the solution is clear; the addition of a little sodium sulphate seems to ensure more successful precipitation by the iron. The solution is filtered while still warm and the bulky iron precipitate is removed from the filter-paper, washed and filtered through the same paper. The washing is carried out three times. The reducing substance in the clear fluid thus obtained is estimated by the method of Hagedorn and Jensen. The estimation is usually done in triplicate, sometimes in quadruplicate.

The clear solutions obtained by these two methods obviously contain no substances that are not soluble in water. It is unlikely that any breakdown

of larger molecules either by enzyme action or by acid hydrolysis could occur during the course of the extraction processes under the conditions described.

The actual values obtained are shown in Table I. The values given by the alcohol extraction method are always higher than those given by the trichloroacetic acid method. The individual variation is obviously considerable. As the two methods show a definite parallelism it seems unlikely that this variation is due to any error of technique.

Glycogen estimations have not as yet been attempted.

Table I.

No.		Wt. brain taken g.	Trichloroacetic acid method. Reducing sub. mg./100 g. brain	Alcoholic method. Reducing sub. Unhydrolysed mg./100 g. brain	Alcoholic method. Reducing sub. Hydrolysed mg./100 g. brain
	Normal				
1	Young	3	100	122	257
2	,,	4	113·8	123·4	216
3	,,	3	109·15	121·3	228
11	,,	2·5	89·1	117	176
13	,,	3	84	117·26	180·6
15	,,	3	76·8	—	189
6	Adult	4	70·6	94·8	152·6
8	,,	4	77·6	—	—
	Insulin				
4	Young	3·5	78·7	93·4	196
5	,,	3	80·03	89·95	186
7	Adult	4	69·5	94·7	146·3
9	,, (C)	4	62·8	84·7	149
10	,,	4	—	90·7	159·4
14	Young	2·5	81·47	104·8	188·2

"C" = convulsed.

The reducing values always rose considerably on hydrolysis, thus indicating the presence of some disaccharide, or polysaccharide, or of some hydrolysable compound yielding a reducing substance.

It will be seen that on an average the rabbits which had received doses of insulin gave somewhat lower values than the normal rabbits, and one or two of the lowest values were given by the former group. It does not seem, however, that this fall in the content of reducing substance always occurs, and it is therefore not likely to be of much importance in accounting for the occurrence of the convulsions.

The extent of the individual variation made comparison of the normal and "insulin" rabbits very difficult. We found, however, that rabbits taken from the same litter gave very constant results, and we adopted the plan of taking both experimental and control animals from the same litter. The figures in Table II show that much clearer results can be obtained in this way; they do not indicate any fall in the amount of reducing substance as a result of treatment with insulin.

To avoid confusion with any possible result of the convulsive muscular movements themselves, the "insulin" rabbits were usually killed in the flaccid condition which immediately precedes the actual convulsions.

Table II.

No.	Litter A	Wt. brain taken g.	Trichloroacetic acid method. Reducing sub. mg./100 g. brain	Alcohol method. Reducing sub. Unhydrolysed mg./100 g. brain	Alcohol method. Reducing sub. Hydrolysed mg./100 g. brain
13	Young normal fed	3	84	117·26	180·6
14	Young insulin starved	2·5	81·47	104·9	188·2
15	Young normal starved	3	76·8	—	189·47
	Litter B				
	Young insulin stood (C)	3	—	145·5	207·3
	Young normal stood	3	—	127·7	187·3
	Young insulin (C)	3	—	137·3	205·6
	Young insulin	3	—	146·88	208·1

"C" = convulsed.

"Stood" indicates that the brain was allowed to stand for 24 hours at room temperature before extraction.

In view of the fact that brain tissue is capable of breaking down large quantities of glucose, and considering also that the convulsions coincide with a low blood sugar, and can be cured by administration of glucose, it seems curious that the reducing substance in the brain tissue should be left untouched when the blood sugar is low.

One possible explanation which suggested itself was that the reducing substance which is extracted from the brain was not glucose.

In order to test this, estimations of the lactic acid content of brain tissue immediately after removal of the brain were carried out, and the figures compared with those given by estimations of the lactic acid content of brain tissue after incubation or standing at room temperature. The method used was that of Meyerhof [1920]. It was necessary to substitute centrifuging for the original filtration through filter-paper. The values given by the distillations are quoted without correction, as comparative rather than absolute values were required.

The values for lactic acid in the brain immediately after death ("resting" lactic acid), expressed as mg. per 100 g. brain tissue, are all very much alike. These values are given in Table III.

Table III.

No.	Rabbit	mg. lactic acid per 100 g. brain	Description	
9 A	Normal	65·29	Resting	Halves of same brain
9 B	,,	62·74	Stood 24 hours room temp. unchopped	
14	,,	98·18	Resting	Animals of same litter
15	,,	84·15	Stood 24 hours room temp. chopped	
18 A	,,	88·73	Resting	Halves of same brain
18 B	,,	85·79	Stood 24 hours room temp. unchopped in N_2	
19 A	,,	73·61	Resting	
19 B	,,	86·18	Stood 24 hours room temp. chopped in cyanide Ringer p_H 8·4	,, ,,
17 A	Insulin	35·29	Resting	
17 B	,,	21·48	Stood 24 hours room temp. unchopped in N_2	,, ,,
20 A	,,	20·90	Resting	
20 B	,,	24·64	Stood 24 hours room temp. chopped in cyanide Ringer p_H 8·4	,, ,,

As far as we could discover, the lactic acid content did not rise on incubation or on standing at room temperature. The brains were either allowed to stand unchopped in the air, or chopped in ordinary Ringer solution containing cyanide ($N/65$ and in some cases $N/650$ KCN).

In one case Ringer solution of p_H 8·4 was used in order to favour the formation of lactic acid as far as possible. It was usually assumed that a whole or half-brain standing unchopped in the air would be lacking in oxygen, in which case any lactic acid formed would not be oxidised. With the intention of testing this point two experiments were carried out in which the half-brains not used for "resting" determinations stood for 24 hours in an atmosphere of nitrogen.

In no case was any detectable rise in the lactic acid content of the brain tissue found as a result of standing or incubation, except possibly in the two half-brains (Table III, 19 B, 20 B) which stood chopped in alkaline Ringer solution. Even here, the rise was so slight as to be almost within the limits of error.

It seems more than probable that, during the life of the animal, glucose diffuses into the brain from the blood, and is present in the brain in estimable amounts, but that in the few minutes elapsing between the death of the rabbit and the freezing of the brain, practically the whole of this is metabolised. Some of this glucose would thus appear as "resting" lactic acid.

When the tissue is allowed to stand unchopped, or to stand chopped, in a Ringer solution of about the p_H of the body, the lactic acid content does not rise above the "resting" level. If it stands chopped in a Ringer solution of p_H 8·4, a trace of additional lactic acid seems to be formed. Probably, therefore, a small amount, but only a very small amount, of glucose remains in the brain after the death of the animal, in which case, a trace, at most, of the reducing substance estimated in the brain after death is glucose.

We have, as yet, had no opportunity of studying the fate of the reducing substances when the brain is kept at p_H 8·4, but the conclusion that there is no further formation of lactic acid on standing is supported by the fact that neither a decrease of the reducing substance in the brain tissue on incubation or on standing, nor any hydrolysis of the hydrolysable fraction can be shown to occur. To confirm this an experiment was performed using two halves of one brain for sugar estimations and two halves of another brain for lactic acid estimations (see Table IV). The two rabbits were of one litter, and the experiments were carried out on the same day. One-half of each brain was used as quickly as possible, in this case about 6 minutes after the death of the rabbit; the other half of each brain was, as usual, covered over to prevent drying, and then left unchopped for 24 hours at room temperature.

If, on the other hand, glucose is supplied to the brain tissue, that is to say if the tissue is chopped and placed in Ringer solution of p_H 7·4 containing glucose but no phosphate, considerable amounts of lactic acid are formed (see Table V).

Table IV.

			mg. per 100 g.
Rabbit 9	9 A	Lactic acid. "Resting" value	65·29
	9 B	Lactic acid. Value after 24 hours room temp. ...	62·74
Rabbit 10	10 A	Reducing substance. "Resting" value; unhydrolysed	107·3
	,,	Reducing substance. "Resting" value; hydrolysed ...	165·7
	10 B	Reducing substance. Value after 24 hours room temp.; unhydrolysed	105·6
	,,	Reducing substance. Value after 24 hours room temp.; hydrolysed	169·8

Reducing substance calculated as glucose.

Table V.

No.		mg. lactic acid per 100 g. brain
8 B	Stood 24 hours room temp. in Ringer (N/65 KCN)	76·60
8 A	Stood 24 hours room temp. in Ringer (N/65 KCN) + 0·25 % glucose	207·70
21 A	Stood 24 hours room temp. in Ringer (N/65 KCN)	62·70
21 B	Stood 24 hours room temp. in Ringer (N/65 KCN) + 0·25 % glucose	293·30

In every case p_H of Ringer = 7·4.

It seemed that some knowledge of the actual reducing substances present would be very useful at this point, but so far the information obtained has been somewhat scanty, and the matter is still under investigation.

Preliminary experiments indicate the presence of a definite amount of pentose in the aqueous solution obtained after alcoholic extraction (and probably also after trichloroacetic acid extraction) of the tissue. The pentose tests are given very strongly by the solution, whereas Van der Haar's [1918] test, said to be specific for glycuronates, is negative. The method described by Tollens [1910] for the estimation of pentoses, has so far been attempted in one case only. The value obtained in this one case was about 20–30 mg. pentose in 100 g. brain tissue. That such an appreciable amount of pentose is present in our solutions is surprising, since the pentose or pentose-containing substance estimated must be soluble in water, and it is difficult to see how any hydrolysis of substances such as nucleic acids could occur during the extraction process. Work bearing on this question is still continuing.

We have so far no information regarding the nature of the hydrolysable substance. Estimations of the inorganic phosphates before and after hydrolysis have been carried out, and seem to show that the hydrolysable substance is not a phosphate compound, since there is no parallel rise of inorganic phosphates on hydrolysis. Again, the numerical relationship of the reducing values before and after hydrolysis (the method of Hagedorn and Jensen being used throughout) is not such as would be found were the hydrolysable substance a disaccharide such as maltose or lactose. By the Hagedorn and Jensen method maltose and lactose are estimated as about 75 % of their weight of glucose—much higher than they are estimated by copper reduction methods.

Actual figures are as follows:

Amounts of maltose and lactose estimated as 75 mg. before hydrolysis are estimated after hydrolysis as 102 and 91·4 mg. respectively.

Reducing substance from brain (alcohol method). Estimated before hydrolysis as 84·7 mg., and after hydrolysis as 144·9 mg.

Thus the rise in the reducing value after hydrolysis of the substance extracted from brain is far larger than the rise in value of maltose and lactose after hydrolysis.

The hydrolysable substance is also extracted by the trichloroacetic acid method, but the formation of chloroform from the trichloroacetic acid on boiling makes hydrolysis of solutions containing much of this acid inconvenient.

Finally, a comparison was made between the "resting" lactic acid values of normal and "insulin" brains, and also between the values obtained after incubation or standing. It will be seen that the "resting" values of the "insulin" brains are much smaller than those of the normal brains (see Table VI). This supports the view that there is a shortage of glucose in the brain of a rabbit which is on the verge of insulin convulsions.

It is, however, always possible that the low values are due to an increased oxidation of lactic acid as a result of the administration of insulin. It is hoped that this question will be decided by a study of the relationship between the level of glucose in the blood and the amount of lactic acid in the brain after death.

Table VI.

No.		mg. lactic acid per 100 g. brain	Remarks
5	Normal	90·16	Resting
9 A	,,	65·29	,,
14	,,	98·18	,,
18 A	,,	88·73	,,
19 A	,,	73·61	,,
19 B	,,	86·18	Stood 24 hours room temp. $N/650$ KCN Ringer p_H 8·4
1	,,	72·74	Stood 24 hours room temp. chopped; cyanide Ringer
2	,,	85·53	Stood 24 hours room temp. unchopped
4	,,	75·78	Stood 3 hours room temp. unchopped
8 B	,,	76·60	Stood 24 hours room temp. chopped in cyanide Ringer
9 B	,,	62·74	Stood 24 hours room temp. unchopped
15	,,	84·15	Incubated 4 hours unchopped
18 B	,,	85·79	Stood 24 hours in N_2 at room temp. unchopped
6	Insulin	38·70	Resting
7	Insulin (C)	28·16	,,
17 A	Insulin	35·29	,,
16	,,	42·70	,,
20 A	,,	20·90	,,
17 B	,,	21·48	Stood 24 hours room temp. unchopped in N_2
20 B	,,	24·64	Stood 24 hours room temp. chopped in cyanide Ringer

Averages

Normal		Insulin	
Resting	Stood or incubated	Resting	Stood or incubated
83·19	78·66	33·15	23·06

"C" = convulsed

It appears from these preliminary experiments that the reducing substance in brain tissue which is estimated by our methods, and probably, also, by the

methods employed by Takahashi and Cori, is not glucose. The brain is therefore mainly dependent on the blood circulating through it for its supply of glucose; and when the blood sugar level has fallen low, the brain may well be supposed to suffer from glucose starvation. The fact that the reducing substance found in the brain is not necessarily abnormally low at the onset of the convulsions is not significant, since this reducing substance is not glucose.

SUMMARY.

1. The amount of reducing substance which can be extracted from the brains of normal rabbits, and from those of rabbits treated with insulin, has been investigated. Two different methods have been used simultaneously for extraction, and reducing substance has been estimated by the method of Hagedorn and Jensen.

2. It is concluded that there is no marked change in the amount of reducing substance as a result of insulin administration. This confirms the findings of Cori and of Takahashi.

3. The reducing substance of brain is not capable of giving rise to the formation of lactic acid, although, in similar conditions, abundance of lactic acid is formed by the brain from added glucose. Further, the reducing substance consists partly of a pentose, and partly of a substance, the reducing power of which is greatly increased after acid hydrolysis. Probably little or none of it is glucose.

4. Determinations of "resting" lactic acid on the brains of normal and of "insulin" rabbits show a greatly reduced lactic acid formation in the latter case. Neither in "normal" nor in "insulin" brains is there an increase in lactic acid formation over the "resting" value after standing or incubation at body p_H.

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LXXIV. THE PERSISTENCE OF VITAMIN A IN PLANT TISSUES.

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It was recognised at a very early stage of research on vitamin A that those plant tissues which have been engaged in the process of carbon assimilation contain relatively large quantities of this factor and that resting tissues such as seeds contain very small quantities, if any, of it. Its absence from etiolated leaves (*i.e.* those which have never been exposed to light) has been shown, and also its presence in all plant tissues so far examined which contain lipochromes and which have also been exposed to the light. Of underground structures, the only ones found to contain more than the merest trace are the carrot and the yellow sweet potato, each of these also containing lipochromes [Steenbock and Gross, 1919; Steenbock, Sell and Boutwell, 1921; Steenbock and Sell, 1922; Coward, 1923, 2]. Comparisons between different green vegetables or salads have sometimes been drawn with regard to the vitamin content, but apparently very little consideration has been paid to the possible differences of vitamin content of leaves of the same plant grown or preserved under different conditions. Hart, Steenbock, Hoppert and Bethke [1922] drew attention to a difference in vitamin content of two different samples of hay but ascribe it to the method of curing rather than to a difference in the hay on cutting. Drummond and his co-workers [1924] had also noticed a very marked difference in the vitamin A content of butters made from the milk of cows fed at the beginning of the experiment on a sample of hay which had been cut when growing vigorously and, later, on one which had been cut when it was beginning to wither. That this difference in the butters could be ascribed largely to a difference in the vitamin content of the two samples of hay was shown in the results of a feeding test of the hays themselves on rats. An isolated observation by Coward and Drummond [1921] that shoots of *Tradescantia* kept in water-culture solution in the dark for some 8 weeks had lost only very little of their vitamin activity, and a further observation by Coward [1923, 1] that sunflower seedlings exposed to the light and then kept in the dark for (*a*) 1, (*b*) 8 days had apparently lost none of their activity, seemed to make it desirable to investigate the question of the persistence of vitamin A in plant tissues more fully. It was decided to use wheat seedlings

for this purpose because they could be grown easily under laboratory conditions and also to compare green and yellowing autumn leaves, for supplies of these growing under natural conditions could easily be obtained.

Experimental.

Wheat seeds (Carter's Red Stand-up) were set in pots on successive days so that seedlings of the same age should be available for feeding on each day of the test. They were kept in the dark until 8 days old, transferred to the light as the experiment demanded and the shoots cut between 9 and 9.30 a.m. each morning. These were carried in a covered box to the animal room and were eaten immediately by the rats. The basal diet was that in general use in these laboratories. It consisted of:

Caseinogen, heated in thin layers 24 hours, 105°	20 g.
Starch (rice)	50 „
Hardened bean seed oil	10 „
Salt mixture	5 „
Yeast extract	5 „
Lemon juice	5 cc.

The daylight to which the shoots were subjected was never very brilliant, the experiment being carried out in July and August of 1924. It did not vary much from day to day, but any variations would be felt equally in the different experiments as they were all run concurrently with this purpose in view. At first the test was carried out on one shoot, then the dose was doubled and finally three shoots were used to obtain continued growth.

A. *Etiolated shoots.*

Eight days after the seeds were set, the shoots had grown to a height of about 3 inches. No evidence of even traces of vitamin A was obtained by giving one, two or three shoots to each test rat per day (Fig. 1 *a*).

B. *Effect of one day's exposure to light.*

Each day a pot containing 8 days old seedlings was exposed to such daylight as there was for 1 day. The shoots turned green and produced a small amount of vitamin A as shown in the curves of Fig. 1 *b*.

C. *Effect of absence of light after exposure to it.*

The pot of seedlings which had been exposed to light for 1 day was brought back into the dark room.

(1) First 4, then 8 and later 12 shoots were cut off close to the root and distributed equally between four small test tubes each containing 1 cc. tap water, the cut ends only standing in the water. After 24 hours the water from each test tube was given to a test rat by a pipette to see whether the vitamin had diffused into the water. The curves in Fig. 1 *c* show that there was apparently no diffusion.

(2) The shoots from the above experiment were given to other rats which only showed really satisfactory growth when the highest dose of three shoots was given (Fig. 1 *d*).

(3) Shoots which had been left on the plants in the pot during 24 hours in the dark were also tested, and caused resumption of growth in rats, though not such a satisfactory response as was given by the shoots which had been standing in water. It is doubtful whether the difference is great enough to justify a conclusion that the vitamin had travelled down to the living root more readily than it had diffused into the water (Fig. 1 *e*).

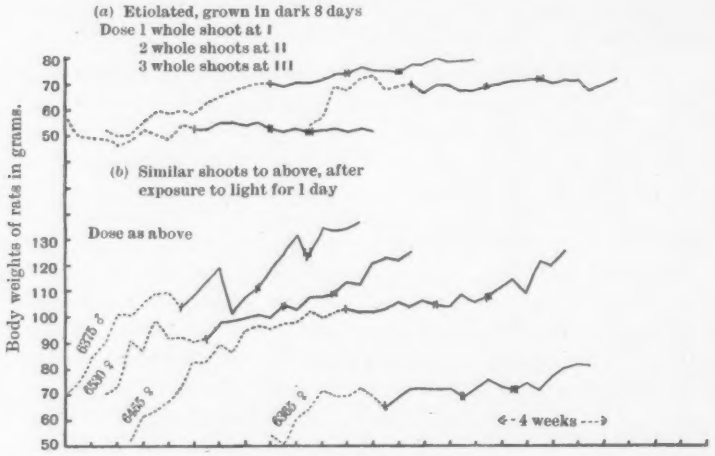

Fig. 1 *a*, *b*. Wheat seedlings.

(4) Other shoots of the same series were tested after the plants had been kept for 16 days in the dark. During this time, they had grown to about three times their original length, a second leaf had grown out of each shoot and the whole had become nearly colourless. A little green colour was left in the oldest leaf, and though the tips of the leaves had become dry and withered, for the most part the leaves were still fairly fresh. That the vitamin had not been destroyed during this time is shown in Fig. 1 *f*.

D. *The vitamin content of the withered leaf.*

A parallel series of seedlings was set and grown in the open air and daylight. The first-formed leaf was tested when the plants were 13 days old and was found to give growth. Similar first-formed leaves were tested when they were 43 days old and quite dried up and withered. They gave no growth (Fig. 2 *a* and *b*).

Hence it appears that the vitamin is not used up in any process carried on in the dark, and is not destroyed until the leaf really dies.

Confirmation of this point was sought in a comparison of the green and yellowing autumn leaves of various trees, *e.g.* lime, plane and poplar, and

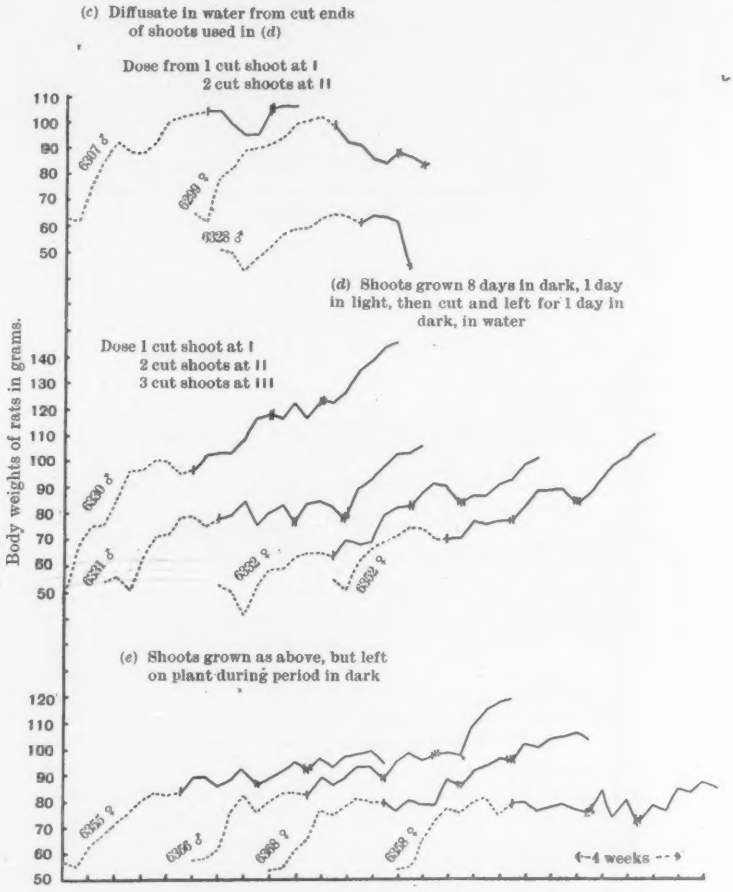
(c) Diffusate in water from cut ends of shoots used in (d)
Dose from 1 cut shoot at I
2 cut shoots at II
6307 ♂
6329 ♀
6328 ♂
(d) Shoots grown 8 days in dark, 1 day in light, then cut and left for 1 day in dark, in water
Dose 1 cut shoot at I
2 cut shoots at II
3 cut shoots at III
6330 ♂
6331 ♂
6332 ♀
6332 ♀
(e) Shoots grown as above, but left on plant during period in dark
6355 ♀
6356 ♂
6368 ♀
6358 ♀
4 weeks
Body weights of rats in grams.

Fig. 1 *c*, *d*, *e*. Wheat seedlings.

also of watercress and cabbage. The tree leaves that were used, both green and yellow, were collected fresh each day from those that had been blown down during the previous day or two. The greenest parts were used for the "green" test, the yellowest (fresh) parts for the "yellow" test. The dead, withered leaves of the lime were also tested. The watercress supply was renewed twice

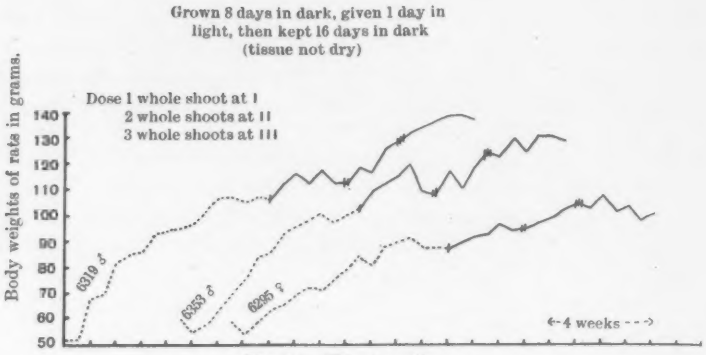

Fig. 1 *f*. Wheat seedlings.

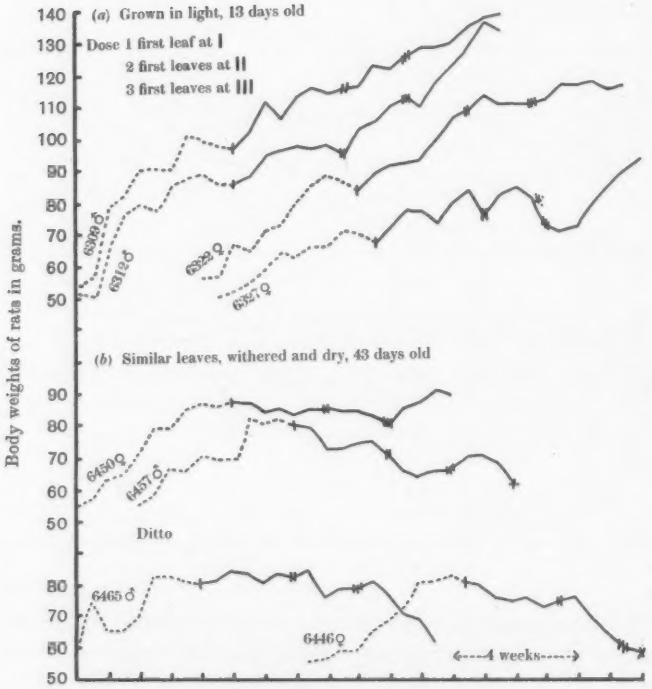

Fig. 2. Wheat seedlings.

* This rat had both legs broken accidentally about this date, which may have accounted for a temporary fall in weight.

a week and a comparison made between the fresh green leaves of the shoots and the yellow ones that had changed colour since coming into the laboratory, during which time the shoots had been freely supplied with fresh tap water. In the case of the cabbage, the comparison was made between the strong green outer leaves and similar leaves that had turned yellow on the plant while its stem was standing in water in the laboratory. It was necessary to renew this supply twice a week in order to ensure the testing of yellowed

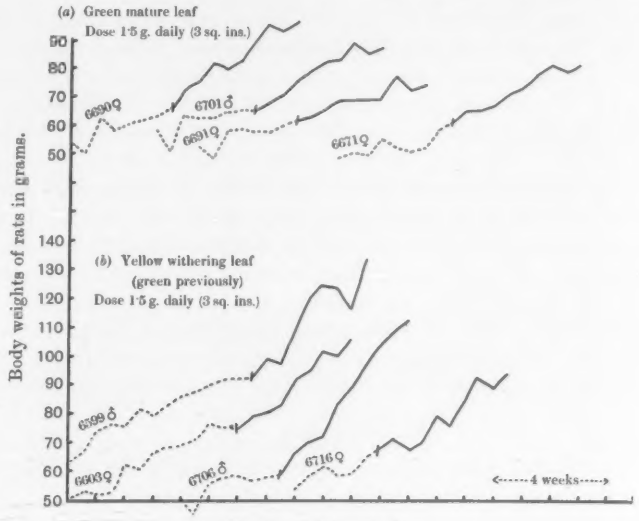

Fig. 3. Cabbage leaf.

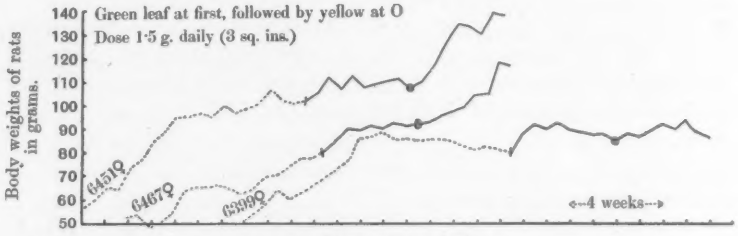

Fig. 4. Plane leaf.

leaves that had grown under the same conditions as the green ones. In view of the fact that light is a controlling factor in the formation of vitamin A, the tests were carried out with as nearly as possible equal *areas* of leaf surface, *i.e.* about 3 square inches daily, rather than equal *weights*.

Two typical results of these feeding tests are seen in Figs. 3 and 4. In each case the growth obtained with the yellow leaves was at least as good as

that obtained with the green; it appears to be even better, but with such small numbers of animals in each test it is difficult to say whether the observed difference has any real significance. The result with the plane leaves, however, appears to be quite definite.

Withered lime leaves which had passed through the clear yellow stage, fallen from the tree and died, gave no growth.

Thus, it would appear that vitamin A is not used up in the yellowing autumn leaf until it actually dies.

One test was carried out on the calyx cups of the winter cherry (*Physalis Alkekengi*). The specimens used had been picked when quite fresh, dried and kept for 12 months. A dose of 0·1 g. per day was sufficient to cause resumption of growth in test animals. A similar test made previously [Coward, 1923, 2] on a different sample immediately after drying had given good growth also on a dosage of 0·1 g. daily.

It is interesting to examine these results in the light of the work of Steenbock and Nelson [1923] on the necessity of the antirachitic factor for growth. No attempt was made to supply the test rats with the antirachitic factor other than whatever may have been present in the shoots, but it may safely be assumed that where continuous growth was obtained (as with the three shoots in many cases), particularly after a long preparatory period (whether this was intentional or not), it must have been the growth-promoting vitamin A which the shoots supplied.

Summary.

1. Vitamin A is not used up in any process carried on by living plant tissue in the dark.

2. It appears to increase when a leaf loses its green colour and becomes yellow.

3. It is completely destroyed when the leaf dries up and dies.

4. It does not diffuse appreciably into water from the cut ends of shoots.

The thanks of the writer are gratefully offered to the Royal Society and to the Medical Research Council for grants which jointly defrayed the cost of these experiments.

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LXXV. STUDIES ON XANTHINE OXIDASE.

V. THE FUNCTION OF CATALASE.

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(*Received May 1st, 1925.*)

Although the enzyme catalase has been known for a considerable time, its biological function has never been definitely settled. It was first suggested by Loew [1902] that it had a protective action, and that its function was to prevent the accumulation in the cell of the hydrogen peroxide formed during the oxidation processes connected with cell respiration. It is well known that hydrogen peroxide is extremely toxic to most enzymes, and its presence in the cell, except in very small concentrations, would rapidly bring about their destruction. Loew suggested that the presence of catalase prevented this by keeping the peroxide concentration down to a low level. This view has been fairly generally held, but so far without having been established by definite experimental evidence except in the case of certain bacterial systems. McLeod and Gordon [1922] showed that pneumococci with a free supply of oxygen produced hydrogen peroxide in amounts sufficient to inhibit their growth; but if catalase was added they were able to grow much more vigorously than in its absence, so that Loew's view is thus supported at any rate as far as bacteria are concerned. In the present paper evidence is given to show that catalase does actually have a protective function in animal tissues.

In the second paper of this series [Dixon and Thurlow, 1924, 2] the results have been given of investigations on the xanthine oxidase by means of the methylene blue technique. Morgan, Stewart and Hopkins [1922] also carried out a number of experiments on this enzyme by measurements of oxygen uptake, and it was during an attempt to extend these investigations that the facts described in the present paper came to light.

The preparation of xanthine oxidase previously described [Dixon and Thurlow, 1924, 1] was employed for the investigation. It is very suitable for the purpose, as it probably contains no other enzymes and shows no oxygen uptake in the absence of hypoxanthine, xanthine, etc. The oxygen absorption was measured by means of the Barcroft differential apparatus of the type used by Morgan, Stewart and Hopkins. The flasks of this apparatus were of a flattened form which exposed the fluid in a thin layer to the air above it so as to allow very rapid diffusion of oxygen into the liquid. Under these conditions the rate of oxygen absorption depends only on the rate at which it is used up by the enzyme, and is not limited by the rate of diffusion into

the liquid. In setting up each experiment solutions of the same composition were added to both flasks, but the hypoxanthine was added to one flask only. This ensured that each experiment was properly controlled. The experiments were in all cases carried out at a temperature of 40° and at p_H 7·6.

On investigating the effect of a change in the concentration of the enzyme on the rate of oxygen uptake, using a constant amount of hypoxanthine, it was found that the rate of the reaction was *not* directly proportional to the enzyme concentration, as was found to be the case with the methylene blue technique. Moreover, the total amount of oxygen taken up by the system was not always independent of the enzyme concentration. As long as the xanthine oxidase is present in excess the total oxygen uptake corresponds with the calculated amount necessary to oxidise the hypoxanthine (or xanthine) to uric acid. When, however, the concentration of enzyme used in the experiment is reduced, a point is reached where the oxygen uptake varies with the amount of enzyme taken. These facts can be seen from Fig. 1, where the results of a typical experiment are given.

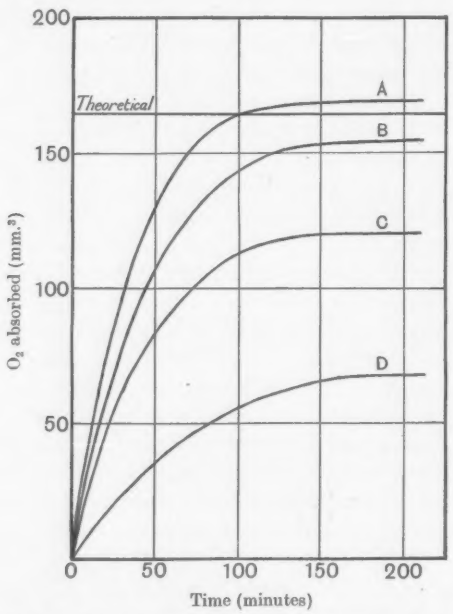

Fig. 1. Oxygen absorption by hypoxanthine with varying amounts of xanthine oxidase.

The solutions were made up as follows:

	A	B	C	D
	cc.	cc.	cc.	cc.
Oxidase solution	2·0	1·5	1·0	0·5
p_H 7·6 buffer	0·5	1·0	1·5	2·0
Hypoxanthine (2 mg. per cc.)	0·5	0·5	0·5	0·5

The curves suggest that the enzyme is being gradually destroyed during the course of the reaction. In order to test whether this was the case the contents of the flasks of the Barcroft apparatus were transferred at the conclusion of the experiment to vacuum tubes, and tested anaerobically with methylene blue and a little fresh hypoxanthine. By comparing the reduction-time given by an enzyme solution which has brought about the oxidation of the hypoxanthine with that given by the solution in the control flask of the same apparatus it is possible to determine approximately how much of the enzyme has been destroyed during the oxidation. By this method it was found that in experiments B, C and D the enzyme had been totally destroyed during the oxidation, whilst in experiment A there had been considerable, though not complete, destruction. It follows therefore that in order to make sure that the oxidation of the hypoxanthine shall proceed to completion it is necessary to use an amount of enzyme sufficient to ensure that some of it shall be left undestroyed at the end of the reaction.

The experiment given in Fig. 1 was repeated, using milk instead of the xanthine oxidase preparation, and similar results were obtained. It is worth noting that the amounts of milk used by Morgan, Stewart and Hopkins in their experiments were not very greatly above the minimum required for complete oxidation of the hypoxanthine, so that it is almost certain that the greater part of the oxidase had been destroyed by the end of their experiments.

These facts show that it is almost impossible to interpret curves of oxygen absorption in terms of enzyme dynamics, since the concentration of enzyme is continuously changing during the progress of the reaction. The oxygen-absorption method is therefore not well suited to a quantitative investigation of the dynamics of systems of the type of the xanthine oxidase except for special purposes, and it seems that other methods, such as the methylene blue technique, are more likely to yield trustworthy results.

It can be shown from the methylene blue experiments given in the earlier paper [Dixon and Thurlow, 1924, 2] that the enzyme is not destroyed during the course of the reaction when methylene blue is substituted for oxygen. The effect seems only to occur in the presence of oxygen, and this fact at once suggests the possibility that hydrogen peroxide may be responsible for the destruction. It has been shown by Thurlow [1925] in the preceding paper of this series that hydrogen peroxide is formed during this reaction in appreciable quantities. The writer has found that xanthine oxidase is very sensitive to hydrogen peroxide, and is quickly destroyed even by quite small concentrations. It follows that the destruction of the enzyme during the reaction is indeed to be expected.

We have then in the xanthine oxidase a system, normally occurring in animal tissues, on which catalase should, if Loew's view is correct, exert a protective action. In order to investigate this question experimentally it is desirable to obtain a preparation of catalase which is free from xanthine oxidase and from oxidisable substances. Several methods of preparation were

tried, and the one which was found to give the most satisfactory results in this respect was that of Batelli and Stern [1904]. Catalase preparations made from liver by this method were found not to take up oxygen or reduce methylene blue either in the presence or in the absence of hypoxanthine.

The liver was minced and extracted with twice its own volume of water. The extract was mixed with two volumes of alcohol, and the resulting precipitate filtered off and dried. This precipitate was then extracted thoroughly with three volumes of water with shaking for some hours, and the extract after filtering was mixed with three times its volume of alcohol. The not very voluminous precipitate thus produced was filtered off and dried *in vacuo*. The preparation is readily soluble in water, and in the dry form retains its activity very satisfactorily.

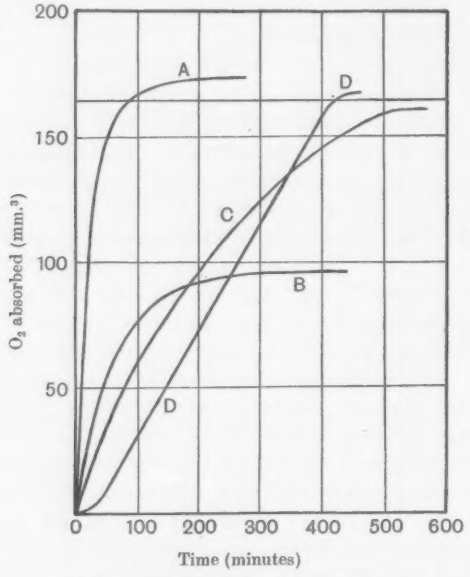

Fig. 2. Effect of addition of catalase.

The solutions were made up as follows:

	A	B	C	D
	cc.	cc.	cc.	cc.
Oxidase solution	2·0	0·5	0·5	0·5
Catalase solution	—	—	0·5 (weak solution)	0·5 (strong solution)
p_H 7·6 buffer	0·5	2·0	1·5	1·5
Hypoxanthine (2 mg. per cc.)	0·5	0·5	0·5	0·5

In Fig. 2 is shown the effect on the oxygen uptake of the addition of catalase. Curves A and B show, as in Fig. 1, the course of the oxygen absorption with a fairly large and a small oxidase concentration respectively; curve C shows

the absorption with the same small amount of xanthine oxidase as in curve B, but with the addition of a relatively small quantity of catalase. It will be seen that the catalase has prevented, at any rate in large measure, the destruction of the oxidase, since the reaction ultimately proceeds to completion even with the small oxidase concentration, though of course at a slower rate than with the higher concentration in curve A.

At the conclusion of the experiment the solutions were tested as before with methylene blue *in vacuo* after the appropriate additions of hypoxanthine, in order to determine how much destruction of the oxidase had occurred. The results showed that in A about 90 % of the oxidase had been destroyed, whilst in B destruction had been complete. In C, however, only about 25 % had been destroyed, and the catalase thus shows a protective action which is very marked considering the small amount added. The same experiment has been repeated with several different preparations of xanthine oxidase and liver catalase, and similar results have invariably been obtained.

If stronger solutions of catalase are used, much more complete protection of the enzyme can be obtained. Curve D shows a typical curve obtained with an amount of catalase several times as great as that used in curve C, with the same amount of oxidase as in curves B and C. It is interesting to note that when the protection becomes more complete the curve of oxygen uptake becomes practically a straight line. When very strong catalase solutions are used, the curves always show a small "latent period" effect. The reaction begins very slowly, and does not attain its full velocity for 20–30 minutes. This effect, which can be seen to some extent in curve D, is at present unexplained.

It will be seen that initially the rate of oxygen uptake is definitely smaller when catalase is present than in its absence. This effect is quite independent of the "latent period," and can be seen in curve C as well as in curve D. Of course, complications are introduced by the fact that the oxidase concentration, when no catalase is present, soon falls, so that the velocity of the absorption falls below that given by the solutions containing catalase; but we may safely assume that during the initial parts of curves B, C and D the concentration of oxidase is roughly the same in all three cases. It is therefore legitimate to compare the velocities during the first part of the experiment. Thus it appears that catalase actually lowers the rate of oxygen uptake. This inhibition is, however, only apparent, and is to be expected. The catalase decomposes hydrogen peroxide in the following way $2H_2O_2 \rightarrow 2H_2O + O_2$, and there is thus an evolution of oxygen gas. The apparent volume of oxygen absorbed, as given by the reading of the Barcroft apparatus, is then the actual volume utilised by the xanthine oxidase *minus* the volume evolved by the catalase. Any catalase action is therefore bound to diminish the observed rate of oxygen uptake. This is probably the true explanation of the phenomenon, and there seems to be no reason to suppose that catalase has any direct action on the xanthine oxidase. The above action, of course, only affects the observed rate

of oxygen uptake; the amount of oxygen ultimately absorbed remains unaltered.

It was stated above that destruction of the oxidase occurred in milk as well as in solutions of the catalase-free caseinogen preparation. The amount of catalase contained in milk thus seems to be insufficient to protect the oxidase. The samples of milk used in the experiment, however, appeared to contain only a small amount of catalase, to judge from rough tests, and it is possible that a more average milk would not show so marked a destruction.

Summary.

1. When the purine bases are oxidised by molecular oxygen in presence of xanthine oxidase as a catalyst, the oxidase is progressively destroyed during the course of the reaction. This fact makes the measurement of oxygen uptake unsuitable as a general method for the investigation of the dynamics of the enzyme.

2. The destruction is due to the hydrogen peroxide which is known to be formed during the reaction.

3. The destruction of the oxidase is prevented by the addition of catalase, which is thus shown to have a protective function in animal tissues.

The writer wishes to express his thanks to Sir F. G. Hopkins for his interest in the work, and also to the Royal Commissioners for the Exhibition of 1851 for the senior studentship held during the period of the investigation.

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LXXVI. BACTERICIDAL ACTION OF SOME ORGANIC COMPOUNDS OF MERCURY.

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(*Received May 6th, 1925.*)

Though many organic compounds of mercury have been prepared, especially during the last few years, and much laboratory and clinical work has been done in ascertaining the possibilities of their application as medicaments, there is as yet a scarcity of systematic information regarding the bactericidal action of such substances, although such knowledge must form the basis on which the use of these products in medicine depends.

The most important work of this kind done so far is that of Schoeller and Schrauth. They showed [1910] that, beginning with a simple compound such as hydroxymercuribenzoic acid HO—Hg—C_6H_4.COOH, there was little change in bactericidal activity when HO— was replaced by bromine or chlorine, a marked diminution on replacement by iodine or cyanogen, still greater reduction on replacement by sulphur, and that activity almost vanished when HO— was replaced by a second benzoyl radical as in mercuridibenzoic acid.

In a second paper [1912] they showed that by the insertion of other substituents in hydroxymercuribenzoic acid the bactericidal action could be markedly influenced. Thus it was increased by halogen or alkyl groups or by a second hydroxymercuri residue and diminished by hydroxyl, sulphonic acid, or amino groups. These observations relate for the most part to new substituents in the *ortho*-position to the carboxyl group, but some evidence was obtained that the orientation of substituents also affected the intensity of bactericidal action, *e.g.* in C_6H_3(COONa)(Hg.OH)(CH_3O) the three substituents in positions 1:2:4 were less efficient than in positions 1:2:6. Two species were used in these experiments, viz. *B. anthracis* and *Staphylococcus pyogenes aureus*. There was little evidence of any specificity in action, but it must be remembered in this connection that all the compounds were not tried on each species.

In a third paper [1916] the same authors dealt with the influence of the same substituents in the absence of the carboxyl group, the latter being replaced by hydroxyl. Here bactericidal action was intensified to start with, the carboxyl group being a weakening factor, but the influence of the substituents already referred to remained the same so far as it was tried, with the

addition of the nitro-group to the augmenting series. The three mercurated isomeric cresols arranged themselves in these trials in the following order of diminishing activity: *meta, ortho, para.*

Reference may also be made to Miss de Witt's work [1921] which relates mainly to the possibility of using organic compounds of mercury in tuberculosis, and covers a large number of substances ranging from mercuric chloride to complex products including chloromercuri derivatives of dyes such as methylene blue, trypan blue, methylene green, etc. The results are expressed in terms of mercury required to inhibit the growth of human tubercle bacilli on melted nutrient agar; and from the present point of view they are chiefly of interest in showing the variation in the amount of mercury (g. per litre) required for this purpose, depending on the organic or inorganic residue with which it is associated, the least effective being mercuric chloride (0·093) and the most active, acetoxymercuriallyl alcohol, $HO.CH_2CH : CH.HgO.Ac$ (0·012), chloromercurimethylene blue (0·014) and chloromercurifluorescein (0·016).

There are numerous other papers on the subject but the foregoing account is sufficient to show the general position.

It occurred to us that valuable information might be obtained by starting with a comparatively simple substance, mercurating it and a suitable selection of its derivatives and determining the bactericidal action of the resulting mercury compounds, keeping throughout to strictly comparable conditions, and using for comparison certain standard mercury compounds, which are already in more or less general use in medicine.

Of the numerous possible starting points for such an investigation we selected benzaldehyde mainly because, when we began this work, no mercurated derivatives of aromatic aldehydes had been made. The substances so far prepared have been described and their constitution discussed elsewhere [Henry and Sharp, 1922, 1924], and in the present paper only their bactericidal action need be discussed. For the elucidation of certain points it proved desirable to make a number of other organic mercury compounds, for example, those of the alkyl phenols, from which we hoped to obtain information as to the effect of replacing the aldehyde group by alkyl groups. Further, for certain clinical trials which became necessary in the course of this work, derivatives soluble in oils had to be made, and this was done by converting the hydroxymercuri compounds into salts of higher fatty acids, such as oleic, myristic, chaulmoogric, etc. These salts, which are described briefly later on, are readily soluble in almond, olive and other oils and such solutions are suitable for chemotherapeutic experiments on animals where aqueous solvents are inadvisable or useless. The bactericidal tests were all made by the methods described below, using *Bacillus typhosus*, Rawlings.

B. typhosus was selected as the test organism in preference to *B. coli* for the reasons put forward by Anderson and McClintock [1912] viz., (1) the greater reliability of the strains in the former organism, (2) the proof that the growth in the medium is actually due to *B. typhosus* and not to accidental

contamination is easily made by using specific agglutination with a high titre serum.

Aqueous solutions. Most of these mercury compounds are insoluble in water but form sparingly soluble sodium or potassium salts and these were used for the tests carried out in water. The solutions were diluted to a suitable series of known concentrations with sterile distilled water and quantities of 5 cc. mixed with 0·2 cc. of a 24 hours broth culture of *B. typhosus*, Rawlings, filtered through paper. This was allowed to interact for 5 minutes at 20° and a 3 mm. platinum loopful taken into 10 cc. of sterile broth and incubated at 37° for 48 hours. The tubes showing growth in the highest concentration were tested with typhoid serum and a control serum for specific agglutination.

Serum solutions. It is now well known that the bactericidal action of many substances is reduced when they are used in presence of serum and to ascertain how this medium affected mercury compounds some trials were made with normal horse serum, which had been passed through a Berkefeld candle and heated at 58° for 2 hours. The serum in this case was used as a diluent in place of sterile distilled water as in the previous test.

Oil solutions. For the reason already stated (p. 514) it was desirable to see how the use of oil as a solvent for oil-soluble mercury compounds affected their use as bactericidal agents and these tests were carried out as follows. The reagents were diluted in sterile olive oil. 5 cc. quantities were tubed and to these varying dilutions, 0·02 cc. of the filtered broth culture was added; the tubes were then closed with sterile rubber corks and the contents vigorously shaken. After 5 minutes' interaction in a water-bath at 20° cultures were taken as before.

Results of Experiments.

The substances are indicated by typical formulae as far as possible, *e.g.* in Table I, substance No. 1 is 3 : 5-dihydroxydimercuri-2-hydroxybenzaldehyde, which is represented thus $C_6H_2[(CHO)(Hg.OH)_2(OH) = 1 : 3 : 5 : 2]$.

From the results given (Table I) it is clear that the introduction of a mercury residue into the molecule of an organic compound enhances the bactericidal action of the latter: thus, whilst a 0·5 % solution of *m*-hydroxybenzaldehyde only kills *B. typhosus* after 4 hours' action, solutions of all the mercury derivatives of *m*-hydroxybenzaldehyde are much more active bactericidal agents, the most potent towards *B. typhosus* being hydroxymercuri-4-nitro-3-hydroxybenzaldehyde (No. 7, Table I) which kills in 5 minutes at a concentration of 0·004 %, whilst the least active are the dihydroxydimercurinitro-3-hydroxybenzaldehydes (Nos. 4 and 8, Table I) which are toxic in 5 minutes at a concentration of 0·06 %. It is noteworthy in this connection that so far from enhancing the bactericidal action, as stated by Schoeller and Schrauth, the introduction of a second mercury residue appears to reduce it, all the dimercuri derivatives of nitro-*m*-hydroxybenzaldehyde (Nos. 4, 6, 8, in Table I) being less active than the monomercuri derivatives (Nos. 3, 5, 7, 9, in Table I).

Table I. *Salts of mercurated hydroxybenzaldehydes, alkylphenols and certain related substances in water.*

Mercurated hydroxybenzaldehydes.

No.	Substance used	Toxicity to *B. typhosus* % kills	Toxicity to *B. typhosus* % fails	Mercury in toxic concentration g. %
1	$C_6H_2[(CHO)(HgOH)_2(OH)=1:3:5:2]$	0·0202	0·013	0·016
2	$C_6H_2[(CHO)(HgOH)_3(OH)=1:3:5:4]$	—	0·03	*
3	$C_6H_3[(CHO)(HgOH)(OH)=1:2:3]$	0·0085	0·0056	0·005
4	$C_6H[(CHO)(HgOH)_2(OH)(NO_2)=1:2:4:3:6]$	0·06	0·05	0·04
5	$C_6H_2[(CHO)(HgOH)(OH)(NO_2)=1:2$ or $4:3:6]$	0·01	0·009	0·005
6	$C_6H[(CHO)(HgOH)_2(OH)(NO_2)=1:2:6:3:4]$	0·02	0·01	0·013
7	$C_6H_2[(CHO)(HgOH)(OH)(NO_2)=1:2$ or $6:3:4]$	0·0038	0·0029	0·002
8	$C_6H[(CHO)(HgOH)_2(OH)(NO_2)=1:4:6:3:2]$	0·06	0·05	0·04
9	$C_6H_2[(CHO)(HgOH)(OH)(NO_2)=1:4$ or $6:3:2]$	0·0095	0·0086	0·005
10	$C_6H_2[(CHO)(HgOH)(OH)(NO_2)=1:5:4:3]$	0·117	0·095	0·06
	Mercurated alkylphenols.			
11	$C_6H_2[(CH_3)(C_3H_7)(HgOH)(OH)=1:4:?:2]$	0·038	0·017	0·021
12	$C_6H[(CH_3)(C_3H_7)(HgOH)_2(OH)=1:4:2:6:3]$	0·033	0·016	0·023
13	$C_6H_3[(C_4H_9)(HgOH)(OH)=1:3:4]$	0·043	0·032	0·024
14	$C_6H_2[(C_4H_9)(HgOH)_2(OH)=1:3:5:4]$	—	0·012	*
15	$C_6H_3[(C_5H_{11})(HgOH)(OH)=1:3:4]$	0·035	0·028	0·018
16	$C_6H_2[(C_5H_{11})(HgOH)_2(OH)=1:3:5:4]$	—	0·022	*
	Miscellaneous mercury compounds.			
17	Hydroxymercuri-*o*-nitrophenol†	0·0021	0·001	0·0011
18	Hydroxymercuri-*m*-nitrophenol	0·005	0·004	0·0028
19	Dihydroxydimercuri-*m*-nitrophenol	0·01	0·009	0·007
20	Hydroxymercuriacetylsalicylic acid†	0·6	0·4	0·26
21	Hydroxymercuridibromofluorescein†	0·3	0·2	0·069

* Strongest concentration possible.

† Commercial preparations issued and used as sodium salts.

In order to confirm this result mono- and di-mercuri derivatives of *m*-nitrophenol were prepared, when it was found that the dimercuri derivative (No. 19, Table I) is only half as active as the monomercuri compound (No. 18, Table I).

On arranging these results as in Table II, to show the percentage of metallic mercury in the concentration toxic to *B. typhosus*, it becomes clear that the bactericidal equivalent, thus expressed in terms of metallic mercury, depends in a marked degree on the nature of the organic residue to which it is attached.

When these effects are considered in detail it is seen that the two groups —CHO and —OH taken together are most effective in the *meta*-position to each other and least effective in the *para*-position. The entrance of a nitro-group when CHO : OH = 1 : 3, enhances the effect in position 4 (No. 7) but exerts no influence in positions 2 or 6 (Nos. 5 and 9) though it should be remembered that these compounds are not strictly comparable since the

position of the mercuri-group is not the same in all three. These results differ from those of Miss de Witt [1921] with mercurated nitrophenols in which the combination HgOH : NO_2 : OH was much more active than HgOH : OH and was at its best with HgOH : NO_2 : OH = 4 : 2 : 1.

Table II.

No. in Table I	Substance	Mercury in toxic concentration g. %
17	Hydroxymercuri-*o*-nitrophenol*	0·0011
7	Hydroxymercuri-4-nitro-3-hydroxybenzaldehyde ...	0·002
18	Hydroxymercuri-*m*-nitrophenol	0·0028
3	2-Hydroxymercuri-3-hydroxybenzaldehyde	0·005
9	Hydroxymercuri-2-nitro-3-hydroxybenzaldehyde ...	0·005
5	Hydroxymercuri-6-nitro-3-hydroxybenzaldehyde ...	0·005
19	Dihydroxydimercuri-*m*-nitrophenol	0·007
6	2 : 6-Dihydroxydimercuri-4-nitro-3-hydroxybenzaldehyde	0·013
1	3 : 5-Dihydroxydimercuri-2-hydroxybenzaldehyde ...	0·016
15	2-Hydroxymercuri-*p*-iso-amylphenol	0·018
11	Hydroxymercuricarvacrol	0·021
12	2 : 6-Dihydroxydimercurithymol*	0·023
13	2-Hydroxymercuri-*p-tert*-butylphenol	0·024
8	4 : 6-Dihydroxydimercuri-2-nitro-3-hydroxybenzaldehyde	0·04
4	2 : 4-Dihydroxydimercuri-6-nitro-3-hydroxybenzaldehyde	0·04
10	5-Hydroxymercuri-3-nitro-4-hydroxybenzaldehyde ...	0·06
21	Hydroxymercuridibromofluorescein*	0·069
20	Hydroxymercuriacetylsalicylic acid*	0·26

* Commercial preparations.

Table III. *Comparative tests in serum, broth and water.*

	Medium						
	Water		Broth		Serum		Mercury in toxic concentration g. %
Substance	Kills %	Fails %	Kills %	Fails %	Kills %	Fails %	
Mercury succinimide	0·001	0·0001	0·001	0·0001	0·1	0·01	Water 0·0005 Broth 0·0005 Serum 0·05
2-Hydroxymercuri-3-hydroxybenzaldehyde 20° (time 5 minutes)	0·0085	0·005	0·05	0·01	0·05	0·01	Water 0·005 Broth 0·03 Serum 0·03
Time 24 hours; 37°	0·0005	0·0001	0·001	0·0005	0·01	0·005	Water 0·0003 Broth 0·0006 Serum 0·006
Time 48 hours; 37°	0·0005	0·0001	0·001	0·0005	0·01	0·005	Water 0·0003 Broth 0·0006 Serum 0·006

In the case of the alkyl phenols the increase of bactericidal activity due to the introduction of mercury residues is much less marked; thus whilst carvacrol, thymol, *p-tert*-butylphenol and *p-iso*-amylphenol are all toxic to *B. typhosus*, under these conditions, at a concentration of about 0·03–0·04 %, the activity of the mercury derivatives (Nos. 11–16, Table I) is of about the same order, but owing to the sparing solubility of the salts of these derivatives in water

the results are perhaps not entirely trustworthy. For the same reason no conclusions can be drawn regarding the effect of replacing the aldehyde group (as in No. 2) by alkyl groups (as in Nos. 14 and 16).

It is clear from the results in Table III that there is a considerable reduction in the bactericidal action of both organic and inorganic compounds of mercury when they are used in serum in place of water: broth however has little or no effect on the activity of mercury succinimide though it reduces the activity of the hydroxymercurihydroxybenzaldehyde from about one-sixth to one-half of that shown in water.

Experiments in oil. As it is desirable for clinical work in some cases to use mercury compounds in oily media, a number of these compounds have been converted into oil-soluble derivatives by converting the —Hg.OH residue into a radical containing the residue of one of the higher fatty acids. Thus if 2-acetoxymercuri-3-hydroxybenzaldehyde is heated at 100° with excess of myristic acid for 1 hour it dissolves and on extracting the cooled product with hot light petroleum (B.P. 40–60°) to remove excess of myristic acid there is left undissolved 2-myristoxymercuri-3-hydroxybenzaldehyde

$$C_6H_3(CHO)(OH)(HgO.CO.C_{13}H_{27})$$

as a colourless indistinctly crystalline powder, melting at 130° and decomposing at 200°. Found Hg = 36·44, calculated Hg = 36·56 %. This product is readily soluble in oils, especially on warming, and in like manner similar derivatives with higher fatty acids can be obtained from the various mercury compounds mentioned in Table I.

A number of such compounds have been made from 2-acetoxymercuri-3-hydroxybenzaldehyde and their bactericidal action on *B. typhosus* has been determined in olive oil. The characters of the principal compounds of this type made are as follows:

2-Chaulmoogroxymercuri-3-hydroxybenzaldehyde $C_6H_3CHO.OH.HgO.CO.C_{17}H_{31}$ M.P. 117°
2-Lauroxymercuri-3-hydroxybenzaldehyde $C_6H_3CHO.OH.HgO.CO.C_{11}H_{23}$ M.P. 131–3°
2-Oleoxymercuri-3-hydroxybenzaldehyde $C_6H_3CHO.OH.HgO.CO.C_{17}H_{33}$ M.P. 110–115°
2-Capryloxymercuri-3-hydroxybenzaldehyde $C_6H_3CHO.OH.HgO.CO.C_7H_{15}$ M.P. 130–1°

The results of the bactericidal tests in oil are as follows:

Table IV. *Higher fatty acid derivatives of 2-hydroxymercuri-3-hydroxybenzaldehyde* (No. 3, Table I).

Acid used and formula of compound	Toxicity to *B. typhosus*		Mercury in toxic concentration
	% kills	% fails	g. %
1. Caprylic acid $C_6H_3(CHO)(OH)(HgO.CO.C_7H_{15})$	0·025	0·01	0·011
2. Lauric acid $C_6H_3(CHO)(OH)(HgO.CO.C_{11}H_{23})$	0·025	0·01	0·0096
3. Myristic acid $C_6H_3(CHO)(OH)(HgO.CO.C_{13}H_{27})$	0·025	0·01	0·0091
4. Oleic acid $C_6H_3(CHO)(OH)(HgO.CO.C_{17}H_{33})$	0·025	0·01	0·0083
5. Chaulmoogric acid $C_6H_3(CHO)(OH)(HgO.CO.C_{17}H_{31})$	0·0076	0·0038	0·0026

The results of these experiments in oil indicate that the activity of 2-hydroxymercuri-3-hydroxybenzaldehyde used in the form of salts of the higher

fatty acids is reduced by about half in this medium, except in the case of the chaulmoogric acid salt where the activity is doubled, due no doubt to the influence of the chaulmoogric acid, which itself exhibits considerable bactericidal action.

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LXXVII. DEHYDROGENATIONS PRODUCED BY RESTING BACTERIA. I.

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It has been shown in earlier communications [Quastel and Whetham, 1924; Quastel, Stephenson and Whetham, 1925] that succinic acid readily reduces methylene blue in presence of resting or non-proliferating bacteria (*B. coli* and *B. pyocyaneus*), and that under similar conditions fumaric acid acts as a hydrogen acceptor. It has been shown, also, that these bacteria activate not only succinic, fumaric, and malic acids, but nitrates and chlorates, these substances effecting oxidations in presence of the bacteria which they do not produce at a perceptible rate in their absence.

A systematic study has now been made of the activating powers of *B. coli communis*, and this communication is intended to be the first of a series dealing with the subject. Numerous substances have been found to exert reducing and oxidising powers in presence of this organism.

Harden and Zilva showed some years ago [1915] that washed *B. coli comm.* will not reduce methylene blue and demonstrated, in a qualitative manner, that it acquired the power of reduction on addition to it of various substances incapable by themselves of effecting the reduction. Many of the substances, which they tested, will be dealt with in detail below.

The technique adopted throughout the work has been described fully in the papers quoted above. The rate of reduction of methylene blue was measured under standard conditions of temperature and hydrogen ion concentration in the presence of the organism and the substance under investigation. The conditions were always strictly anaerobic and such that growth of the organism did not occur. Sometimes the method was varied by using nitrate or chlorate as hydrogen acceptors, the amount of reduction in a given time being determined by an iodometric estimation of the nitrite or chlorite produced.

In the preparation of the emulsion of bacteria, the following points were found important. The organism was grown for 2 days on tryptic broth and separated by centrifuging; it was then washed very thoroughly by repeated centrifuging with normal saline to remove all traces of broth which is itself a powerful hydrogen donator in presence of *B. coli*. The bacteria thus washed were suspended in normal saline solution through which first air and then

nitrogen were bubbled vigorously for a short time to produce a homogeneous suspension and to remove traces of easily oxidisable material. The suspension was then diluted with saline until a small volume (say 0·5 cc.) of the suspension did not reduce 1 cc. of 1/5000 methylene blue solution at p_H 7·2 and temperature 45° within several hours. In the presence of a concentration of sodium succinate equal to $M/100$, this quantity of organism was usually found to reduce the same amount of methylene blue in about 10 minutes. If the time of reduction was much shorter than this, the emulsion of bacteria was further diluted. The emulsion was kept surrounded by ice, since at room temperature there occurs a rapid diminution in activating power and a liberation of reducing substances, probably due to an autolysis of the cells. At 0° these changes are hardly noticeable during the first fortnight, and even after that proceed very slowly. A suspension of *B. coli* which had been kept in the ice-chest for 4 months still exhibited strong activating powers, and a sub-culture on agar gave a heavy growth.

It has been previously shown [Quastel and Whetham, 1924] that the reducing activity of the organism is directly proportional to its concentration, and that the reaction between succinate and methylene blue in presence of the organism has a temperature coefficient of 2·1 between 30° and 60°.

The experimental results given below are typical examples of a large number of observations. The work was done with a number of different strains and suspensions of *B. coli*, and these are referred to as *coli* 1, *coli* 2, etc. The results obtained with *coli* 1 can be compared strictly quantitatively with one another, but only qualitatively with the results obtained with *coli* 2, etc.

The substances under investigation were always brought to p_H 7·2 by means of caustic soda or hydrochloric acid.

The fatty acids.

Table I gives the results obtained with the fatty acid series.

Table I.

Each vacuum tube contained 2 cc. of phosphate buffer p_H 7·4, 1 cc. of 1/5000 methylene blue solution, 0·5 cc. of *coli* 1, and *X* cc. of the fatty acid solution. The volume of solution in each tube was made up to 6·5 cc. with distilled water. The reduction was carried out *in vacuo* at 45°. A control tube showed no reduction in 6 hours.

Fatty acid	*X* cc.	Concentration of fatty acid	Reduction time in minutes
Formic *M*/20	1	*M*/130	2·0
„ *M*/2	1	*M*/13	1·5
Acetic *M*/20	1	*M*/130	No reduction
„ *M*/2	3	*M*/4·3	2·8
Propionic *M*/20	1	*M*/130	No reduction
„ *M*/2	3	*M*/4·3	26·0
Butyric *M*/20	1	*M*/130	No reduction
„ *M*/2	3	*M*/4·3	3·0
Isobutyric *M*/20	1	*M*/130	No reduction
„ *M*/2	3	*M*/4·3	Not completely reduced in 3 hours
Isovaleric *M*/20	1	*M*/130	No reduction
„ *M*/2	3	*M*/4·3	5·0

Formic acid is the most powerful hydrogen donator of the series, and with the exception of some of the sugars is the most active substance we have yet investigated. It has no reducing action *in vitro* on methylene blue, even in strongly alkaline solution.

Table II gives results with the fatty acids obtained with a different strain of *B. coli* (*coli* 2).

Table II.

Procedure exactly as described in Table I. *Coli* 2 used instead of *coli* 1. 2 cc. of an *M*/2 sol. of fatty acid were used in each case yielding a final concentration of *M*/6·5.

Fatty acid	Reduction time in minutes
Formic	1·2
Acetic	9
Propionic	11
Butyric	30
Valeric	23
Isovaleric	25
Caproic	Not reduced
Heptylic	,,
Caprylic	,,
Nonylic	,,

Apart from formic acid, the lower fatty acids are active donators of hydrogen only at high concentrations. Caproic acid exerts a slight activity (see Table III) but higher members than this are apparently inert. A change in the activity of the organism has apparently a far greater effect on the reduction velocities due to the fatty acids than on those due to donators such as formic or succinic acids, which are active at low concentrations.

Fig. 1 illustrates the variation in times of reduction of 1 cc. of 1/5000 methylene blue solution with varying concentrations of acetic, propionic and butyric acids at p_H 7·4 and 45°, always using the same amount of organism (*coli* 1). The propionic acid curve is of a different type from those of acetic and butyric acids. In view of the oxidation of these fatty acids in the body, it is interesting that propionic acid should offer so clear a contrast to acetic and butyric acids in presence of resting *B. coli*.

The reduction time-concentration curves of acetic, butyric and formic acids (Fig. 1) exhibit a feature common to many of the hydrogen donators we have investigated, namely, a rapid decrease of reduction time up to a definite concentration, above which increase in concentration of the hydrogen donator appears to have little or no effect on reduction velocity. At first sight this phenomenon might be explained on the usual grounds by assuming that the enzyme becomes "saturated" with regard to the donator, so that increase in concentration of substrate above this saturation point does not produce greater reaction velocity. There are, however, difficulties in the way of accepting this interpretation. For example, in some cases, *e.g.* propionic acid (Fig. 1) and succinic acid (Fig. 2), such a "critical" concentration is hardly apparent, and the curves do not exhibit the discontinuity which would be expected if the enzyme surface became truly saturated at some particular concentration of the substrate. In a number of cases a definite relationship appears to hold

between the velocity of reduction and the concentration of the hydrogen donator. This is

$$v = k + k' \log c,$$

where v = velocity of reduction (reciprocal of time of reduction),
c = concentration of the hydrogen donator,

and k and k' are constants.

Beyond stating that in a number of cases we have found such a logarithmic relationship to accord with the experimental results, we do not wish to pursue the subject further at present, and hope to deal with it in detail in a later communication.

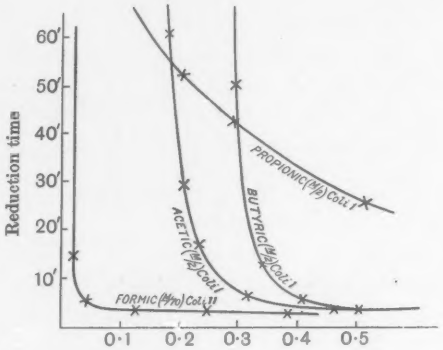

Fig. 1. Concentration of fatty acid

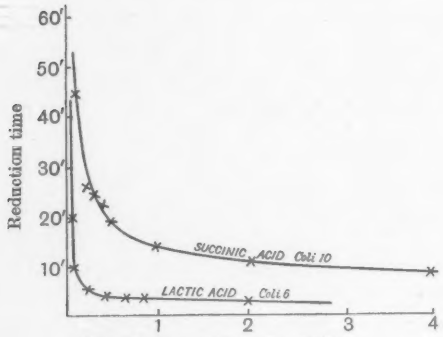

Fig. 2. Concentration ($\times M/140$)

The products of the anaerobic oxidation of the fatty acids in presence of resting *B. coli* have not yet been investigated, but it is clear that in the case of formic acid there is a complete oxidation to carbon dioxide. There is a great increase in p_H during the anaerobic oxidation of formic acid, and carbon dioxide is evolved on treatment with acid.

Pakes and Jollyman [1901] have shown that *B. coli comm.* will decompose formic acid into equal volumes of carbon dioxide and hydrogen. If nitrates are also present in the nutrient medium, nitrites but not hydrogen are produced.

There is some evidence that the fatty acids have a retarding effect on reduction velocity at high concentrations, possibly due to adsorption of the acid on the organism. For instance, isobutyric acid has an optimum concentration as a hydrogen donator, being less active at higher concentrations. The same applies to isovaleric and caproic acids (see Table III).

Table III.

Conditions as in Table I.

Fatty acid	*X* cc.	Organism	Concentration of fatty acid	Reduction time in minutes
Isobutyric *M*/20	1·0	0·5 *coli* 1	*M*/130	No reduction
„ *M*/2	2·0	„	*M*/6·5	145
„ *M*/2	4·0	„	*M*/3·25	Not completely reduced in 6 hours
Isovaleric *M*/2	0·5	0·5 *coli* 3	*M*/26	57
„ *M*/2	1·0	„	*M*/13	49
„ *M*/2	2·0	„	*M*/6·5	Not completely reduced in 2 hours
Caproic *M*/2	1·0	0·5 *coli* 4	*M*/13	74
„ *M*/2	2·0	„	*M*/6·5	Not reduced in 2 hours
„ *M*/2	3·0	„	*M*/4·3	„ „

It is possible that in the case of the higher homologues of caproic acid the reducing activity is masked by greater adsorptive effects which increase with the length of the fatty chain.

The saturated dibasic acids.

Oxalic acid is inert as a hydrogen donator, and malonic, glutaric, and adipic acids may also be regarded as inert, for under the conditions of our experiments these substances have never been found to bring about a complete reduction of methylene blue though they give indications of a slight activity (see Table IV).

Table IV.

Conditions as in Table I.

Acid	*X* cc. of neutralised acid sol.	Concentration of the acid	Reduction time in minutes
Oxalic *M*/20	1	*M*/130	No reduction
„ *M*/2	3	*M*/4·3	„
Malonic *M*/20	1	*M*/130	„
„ *M*/2	3	*M*/4·3	Partial reduction not complete in 4 hours
Succinic *M*/20	1	*M*/130	11·0
„ *M*/2	3	*M*/4·3	4·0
Glutaric *M*/20	1	*M*/130	No reduction
„ *M*/2	3	*M*/4·3	Partial reduction not complete in 4 hours
Adipic *M*/20	1	*M*/130	No reduction
„ *M*/2	3	*M*/4·3	Partial reduction not complete in 4 hours

Oxalic, glutaric, and adipic acids (when mixed with succinic acid) do not retard the reduction due to the succinic acid, but malonic acid has a definite

retarding effect. It is difficult to explain the anomalous behaviour of malonic acid, but there is no doubt as to the reality of the effect (see Table V).

Table V.

Each vacuum tube contained 2 cc. of phosphate buffer p_H 7·4, 1 cc. of 1/5000 methylene blue solution, 0·5 cc. of *coli* 1, 1 cc. of sodium succinate solution *M*/20, 1 cc. of the neutralised dibasic acid (*M*/2), and 1 cc. of water.

Acid	Reduction time in minutes
None (control)	11·0
Malonic	34·5
Glutaric	10·5
Adipic	10·2

The hydroxy acids.

Glycollic acid donates hydrogen only at relatively high concentrations, its behaviour being similar to that of acetic acid. Lactic acid, on the other hand, is an extremely powerful hydrogen donator even at low concentrations. Glyceric acid is active at lower concentrations than glycollic acid but is not as powerful as succinic acid. Tartaric acid is a very feeble donator, whilst citric acid is almost inert (see Table VI).

Table VI.

Conditions as before. *Coli* 5.

Acid	*X* cc. of neutralised acid sol.	Concentration of acid	Reduction time in minutes
Glycollic *M*/20	1	*M*/130	No reduction
„ *M*/2	3	*M*/4·3	3·0
Lactic *M*/20	1	*M*/130	3·0
„ *M*/2	3	*M*/4·3	1·5
Glyceric *M*/20	1	*M*/130	45·0
„ *M*/2	3	*M*/4·3	3·5
Tartaric *M*/20	1	*M*/130	No reduction
„ *M*/2	3	*M*/4·3	21·0
Citric *M*/20	1	*M*/130	No reduction
„ *M*/2	3	*M*/4·3	Not completely reduced in 1 hour

Fig. 3 gives the reduction time-concentration curves for glyceric and glycollic acids (in presence of 0·5 cc. of *coli* 5) and Fig. 2 for lactic acid (in presence of 0·5 cc. of *coli* 6).

The powerful activity of lactic acid compared with the other α-hydroxy acids is very striking. The product of anaerobic oxidation of lactic acid is pyruvic acid; the oxidation of lactic acid in presence of resting bacteria has been fully discussed in a previous communication [Quastel, Stephenson and Whetham, 1925]. The products of anaerobic oxidation of glycollic and glyceric acids have not yet been investigated.

The polyhydric alcohols.

Glycol donates hydrogen only at high concentrations. Glycerol is less active than glycol. Erythritol has no reducing effect (see Table VII).

Table VII.

Conditions as before. *Coli* 5.

Alcohol	*X* cc. of the *M*/2 alcohol solution	Concentration of alcohol	Reduction time in minutes
Glycol	0·5	*M*/26	Not reduced in 2 hours
,,	1·0	*M*/13	71
,,	3·0	*M*/4·3	8·25
Glycerol	1·0	*M*/13	53
,,	3·0	*M*/4·3	30
Erythritol	1·0	*M*/13	No reduction
,,	3·0	*M*/4·3	,,

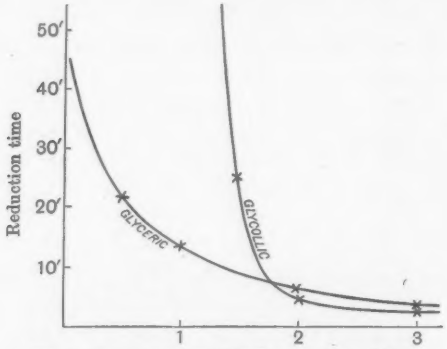

Fig. 3. Concentration of hydroxy acid (× *M*/13)

Glycol, glycerol and erythritol appear to have no retarding effect on reductions due to other substances. The penta- and hexa-hydric alcohols will be discussed later in connection with the sugars.

The glycerophosphoric acids.

α-Glycerophosphoric acid is a much more vigorous donator of hydrogen in presence of resting *B. coli* than glycerol itself, whilst β-glycerophosphoric acid is about as active as glycerol (see Table VIII).

Table VIII.

Conditions as before. *Coli* 5.

Hydrogen donator	*X* cc. of the *M*/2 H donator solution	Concentration hydrogen donator	Reduction time in minutes
Glycerol	1	*M*/13	53·0
,,	3	*M*/4·3	30·0
α-Glycerophosphoric acid	1	*M*/13	2·5
,,	3	*M*/4·3	2·0
β-Glycerophosphoric acid	1	*M*/13	70·0
,,	3	*M*/4·3	20·0

The monohydric alcohols.

The members of this series from methyl to octyl alcohol have been investigated. From butyl alcohol upwards they do not appear to donate hydrogen and all inhibit or retard reductions by other substances. Since these higher alcohols are immiscible with water at ordinary temperatures, they do not lend themselves to quantitative investigation.

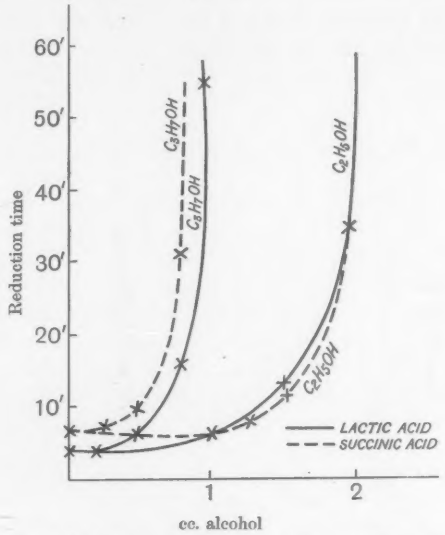

Fig. 4. Effect of alcohols on reduction times of 1 cc. 1/5000 methylene blue by 1 cc. *M*/20 lactic acid and 1 cc. *M*/20 succinic acid.

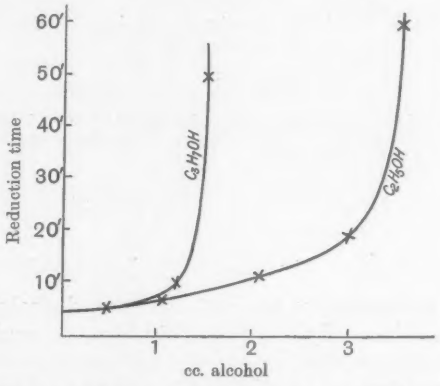

Fig. 5 Effect of alcohols on reduction times of 1 cc. 1/5000 methylene blue by 1 cc. *M*/20 formic acid.

The lowest members—methyl, ethyl and propyl alcohols—all possess the characteristic property of inhibiting the reducing action of hydrogen donators. Total inhibition occurs at definite or critical concentrations of the alcohols, and these concentrations vary with the nature of the hydrogen donator (see Figs. 4, 5, which show the action of varying quantities of the alcohols on the reduction times of a definite quantity of donator, in the presence of 1 cc. of *coli* 7). The critical concentrations of alcohol vary very slightly with the quantity of resting organism used (see Fig. 6) and are independent of the concentration of the donator (see Table IX).

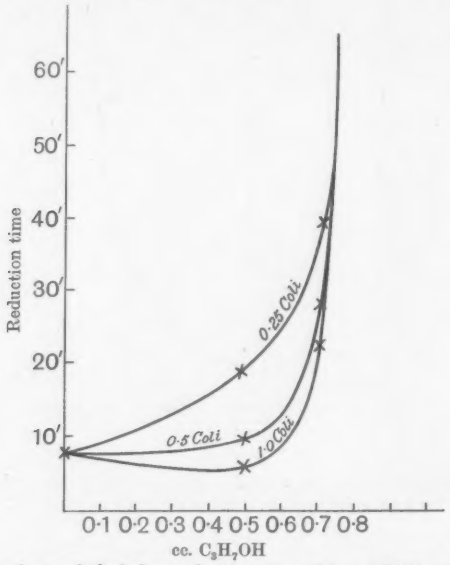

Fig. 6. Effect of propyl alcohol on reduction times of 1 cc. 1/5000 methylene blue by succinic acid.

Table IX.

Each vacuum tube contained 1 cc. of phosphate buffer p_H 7·4, 1 cc. of 1/5000 methylene blue solution, *X* cc. of 1 % sodium succinate, 0·7 cc. of propyl alcohol and 1 cc. of *coli* 7. The volume was made up to 7 cc. with water.

X cc.	Reduction time
0·5	Not reduced
1·0	,,
2·0	,,
3·0	,,

These critical concentrations are least with propyl alcohol and greatest with methyl alcohol, *i.e.* methyl alcohol inhibits least vigorously.

Besides their property of inhibition, ethyl and propyl alcohols possess the power of donating hydrogen to methylene blue (see Table X). Methyl alcohol does not act as a hydrogen donator.

Table X.

Each vacuum tube contained 2 cc. of *coli* 8*, 1 cc. of phosphate buffer p_H 7·4, 1 cc. of 1/5000 methylene blue solution and *X* cc. of the absolute alcohol. The volume was made up to 7 cc. with water.

Alcohol	*X* cc.	Reduction time in minutes
None (control)	—	23·25
Methyl	0·05	23·0
,,	0·1	23·0
,,	0·5	30·0
Ethyl	0·05	11·0
,,	0·10	10·4
,,	1·5	Not reduced
Propyl	0·05	10·25
,,	0·10	9·75
,,	0·50	10·9
,,	1·00	Not reduced

* *Coli* 8 was a highly reducing organism and is included here to demonstrate the alcohol inhibitions.

The effect of increasing the concentration of alcohol is to produce at first an increasing velocity of reduction. This reaches a maximum and remains constant until a certain concentration of alcohol is reached, when the velocity falls off sharply to zero.

Experiments with the vacuum U-tube (see previous papers) show that the inhibitant effects of the alcohols are not due to oxidation of leuco-methylene blue (as in the case of fumaric acid) but to some physical process, such as adsorption at the surface of the organism. The degree of inhibition is associated with the length and nature of the fatty chain: as shown above, high concentrations of some of the higher fatty acids also bring about a retardation of reducing velocity. The polyhydric alcohols, on the other hand, have no retarding effect.

Inhibitants.

An examination of the inhibiting action of substances other than the monohydric alcohols showed that:

1. Small quantities of cyclohexanol or cyclohexene completely inhibit all reductions by hydrogen donators.

2. Benzene, toluene, phenol, and acetone have definite but relatively slight inhibitory effects (see Table XI).

Table XI.

Each vacuum tube contained 1 cc. phosphate buffer p_H 7·4, 1 cc. 1/5000 methylene blue, 0·2 cc. of 5 % sodium succinate, 0·5 cc. *coli* 9, and *X* cc. of inhibitant. The total volume was made up to 6·5 cc. with water.

Inhibitant	*X* cc.	Reduction time in minutes
Control	—	8
Benzene	0·5	9
Toluene	0·5	14
Phenol (10 % neutral)	0·5	10
Cyclohexanol	0·5	Not reduced in 6 hours
,,	0·2	,,
Cyclohexene	0·5	,,
Acetone	0·5	10

Reducing coefficient.

It is desirable to provide some means for the quantitative comparison of the reducing capacity of the large number of substances which can donate hydrogen to methylene blue in presence of resting *B. coli*. This may be done by comparing one donator with a standard donator—say succinic acid—under similar conditions. The most satisfactory method of comparison is to determine what concentration of a donator will reduce a given quantity of methylene blue in the same time as a definite quantity of the standard donator.

We propose, therefore, to define the *reducing coefficient* of a hydrogen donator with respect to a certain organism as the reciprocal of that (molar) concentration which will reduce 1 cc. of 1/5000 methylene blue solution in presence of a standard amount of the organism in half an hour, succinic acid being taken as a standard and its reducing coefficient being taken as 100. For the determination of the coefficient an amount of freshly prepared organism is taken which will bring about the reduction of 1 cc. 1/5000 methylene blue at p_H 7·2 and 45° in the presence of $M/700$ succinic acid in half an hour.

Table XII gives the reducing coefficients of the substances discussed in this communication. Ethyl and propyl alcohols have not been included, owing to their inhibitory action. The coefficients must only be regarded as approximate and as expressing the correct order of magnitude.

Table XII.

Hydrogen donator	Reducing coefficient (with respect to *B. coli comm.*)
Formic acid	700
Acetic acid	0·95
Propionic acid	0·46
Butyric acid	0·64
Valeric acid	0·5
Caproic acid	Less than 0·4
Caprylic acid ... ... „	0·4
Heptylic acid ... ... „	0·4
Nonylic acid ... ... „	0·4
Oxalic acid ... ... „	0·4
Malonic acid ... ... „	0·4
Succinic acid	100
Glutaric acid	Less than 0·4
Adipic acid ... ... „	0·4
Glycollic acid	0·93
Lactic acid	583
Glyceric acid	5·2
Tartaric acid	0·46
Citric acid	Less than 0·4
Glycol	0·86
Glycerol	0·46
Erythritol	Less than 0·4
α-Glycerophosphoric acid	4·31
β-Glycerophosphoric acid	0·4

SUMMARY.

An account is given of the behaviour of a number of substances as hydrogen donators in presence of resting *B. coli* as the activating source and of methylene blue as the hydrogen acceptor. The following groups of substances are dealt with:

1. Fatty acids.
2. Saturated dibasic acids.
3. Hydroxy acids.
4. Polyhydric alcohols.
5. Monohydric alcohols.

The inhibiting action of the monohydric alcohols and of other substances is discussed. A table is given of the relative reducing powers or reducing coefficients of the hydrogen donators.

We wish to express our indebtedness to Sir F. G. Hopkins for the interest he has taken in this work and the thanks of one of us (M. D. W.) are due to the Medical Research Council for a grant held during this work.

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CONTENTS OF VOLUME XIX, No. 3, 1925.

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PAGE

LI. Haemoglobin in Relation to other Metallo-haematoporphyrins. By R. Hill. (With Plate II) 341

LII. The Specific Action of Plant Ferments. I. The Specific Conditions of Action of Leaf Invertases. By A. V. Blagoveschenski and N. I. Sossiedov 350

LIII. The Specific Action of Plant Ferments. II. The Specific Conditions of Action of Leaf Peptases. By A. V. Blagoveschenski and A. N. Bielozerski 355

LIV. The Physiology of Gastric Urease. By J. M. Luck and T. N. Seth. (With one figure) 357

LV. The Relation between the Metabolism and the Specific Dynamic Action of Amino-acids. By T. N. Seth and J. M. Luck. (With four figures) 366

LVI. On the Methods of Fitting the Formula of Michaelis in Relation to the Effect of Hydrogen Ion Concentration on Enzyme Action to the Data: with some Discussion of the Results. By J. Brownlee. (With two diagrams) 377

LVII. A Study of the Oxidation of the Ammonium Salts of Normal Saturated Fatty Acids and its Biological Significance. By P. W. Clutterbuck and H. S. Raper . . 388

LVIII. Studies on the Physiology of Plain Muscle. III. Comparison of the Reducing Properties of Plain and Striated Muscle. By S. Tsubura 397

LIX. Animal Quinoidine. (Preliminary Paper.) By H. W. Kinnersley, R. A. Peters, and B. T. Squires 404

LX. The Pectin Content of Normal and "Silvered" Apple Leaves. By F. Tutin . 414

LXI. Chemical Investigations of Fruits and their Products. I. Apple Juice as a Source of Sorbitol. By F. Tutin 416

LXII. Chemical Investigations of Fruits and their Products. II. The Fate of Sugar during "Cider Sickness." By F. Tutin 418

LXIII. Oxidising Enzymes. VIII. The Oxidation of Certain Parahydroxy-compounds by Plant Enzymes and its Connection with "Tyrosinase." By M. W. Onslow and M. E. Robinson 420

LXIV. Cholesterol Synthesis in the Animal Body. By H. J. Channon. (With three figures) 424

LXV. Some Phosphorus Compounds of Milk. I. The Presence in Milk of Organic Acid-soluble Phosphorus Compounds. By H. D. Kay. (With one figure) 433

LXVI. Note on the Phosphorus Content of the Blood of Ruminants. By H. D. Kay 447

LXVII. The Influence of Washing upon the Reproductive Rate of *Colpidium colpoda*. By D. W. Cutler and L. M. Crump 450

LXVIII. The Chemical Nature of the Membrane of Potato Cork. By E. Rhodes. (With one figure) 454

LXIX. Studies on the Growth of Yeast. I. The Influence of Volume of Culture Medium Employed. By G. L. Peskett. (With one figure) 464

LXX. Studies on the Growth of Yeast. II. A Further Note on Allelocatalysis. By G. L. Peskett 474

LXXI. Fermentation by Dried Yeast Preparations. By A. Harden. (With four figures) 477

LXXII. The Estimation of Phosphorus and Magnesium. By C. P. Stewart and W. Archibald 484

LXXIII. Contributions to the Study of Brain Metabolism. I. Carbohydrate Metabolism. (Preliminary Paper.) By B. E. Holmes and E. G. Holmes 492

LXXIV. The Persistence of Vitamin A in Plant Tissues. By K. H. Coward. (With four figures) 500

LXXV. Studies on Xanthine Oxidase. V. The Function of Catalase. By M. Dixon. (With two figures) 507

LXXVI. Bactericidal Action of some Organic Compounds of Mercury. By T. A. Henry, T. M. Sharp and E. C. Brown 513

LXXVII. Dehydrogenations produced by resting Bacteria. I. By J. H. Quastel and M. D. Whetham. (With six figures) 520

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